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Proceedings of an International Symposium
Held at Cincinnati, Ohio on December 3-5, 1979
Volume II

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16. ABSTRACT

The purpose of this Symposium was to bring together scientists and engineers from the public and private sectors to discuss their research findings on the health effects of diesel engine emissions and to conclude with a discussion of health risk assessment of diesel exhaust.

The Proceedings are organized into eight main sections corresponding to the format of the Symposium and addressing Physical and Chemical Characteristics of Diesel Emissions, In Vitro Carcinogenic and Mutagenic Effects of Diesel Emissions and Components, Biochemical and Metabolic Effects, Toxicological Effects of Inhaled Diesel Emissions, Mutagenic and Carcinogenic Potency of Extracts of Diesel and Related Environmental Emissions, Mutagenicity of Inhaled Diesel Emissions, Carcinogenic Effects of Exposure to Diesel Emissions, Epidemiological Studies, and lastly a panal discussion on Health Risk Assessment of Diesel Emissions.

The Proceeding papers, in some cases, are more comprehensive than the original presentations in order to provide more thorough coverage of the particular topic. Edited discussions are included with each paper and wherever possible the identity of each questioner is indicated. The list of registrants will enable the reader to contact a speaker for further information.

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Edited By

W. E. Pepelko, R. M. Danner, N. A. Clarke

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INTRODUCTION TO AND EXPERIMENTAL CONDITIONS IN THE GMR CHRONIC INHALATION STUDIES OF DIESEL EXHAUST

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ABSTRACT

A chronic exposure study was initiated to determine the effects of diesel exhaust on the health of experimental animals. For this purpose, test atmospheres of clean air (control) or freshly diluted diesel exhaust at concentrations of 250, 750 and 1500 $\mu g/m^3$ were supplied to four 12.6 m^3 inhalation chambers which housed rats and guinea pigs. Diesel aerosol size and concentration, as well as chamber temperature and relative humidity, were continually monitored and controlled to maintain the exposure dose levels and an environment of 22 ± 2°C and 50 ± 20% relative humidity. The concentrations of CO and NO_x were found to be 5.8 ± 1.0 mg/m^3 and 7.9 ± 1.0 mg/m^3 above ambient in the chamber containing 1500 $\mu g/m^3$ of particulate. Animals were supplied from the chambers, on a random basis, for both intramural and extramural studies throughout the exposure period. The experiment has run uninterrupted for over ten months with mean diesel particle mass concentrations within 2% of the target values.

INTRODUCTION

The General Motors Diesel Engine Health Assessment program is designed to provide data on the potential health effects associated with increased use of diesel engines. Central to the program is the exposure of test animal models to diesel exhaust for the purpose of quantifying their response to particulates. The rationale behind these experimental studies is summarized in the following five program design requirements.

First, an inhalation study should be the one of primary importance in health assessment because inhalation is the major route of exposure for the majority of the population. Other methods of testing, such as skin painting, are valuable for the insights they provide, however, diesel exhaust is released as a fine aerosol and its primary route of entry into the body will be through the lungs.

Second, in any study that analyzes the potential health effects, one should not depend on data obtained by testing only the maximum tolerable dose. Even if this approach might be formally justified for studies on the non-threshold concept of carcinogenicity [1], exposures limited to only the maximum tolerated dose are not acceptable for testing other potential adverse health effects such as irritation and cellular transformation of the respiratory system where the concepts of a well-defined threshold and dose-response curve have been firmly established. Consequently, instead of exposing animals to only one level of diesel exhaust dilution, three concentrations, 1500, 750 and 250 $\mu g/m^3$, were administered as a basis for a potential dose effect curve.

Even the lowest dose, 250 $\mu g/^3$ represents a more than twenty-fold multiple of ambient concentrations that might exist by 1985 under projected sales scenarios. Using this multilevel approach with an extended length of daily exposures permits accumulating exposure doses which are of the similar order of magnitude as compared with studies where only the maximum tolerated dose is used for shorter periods.

The studies are conducted with the expectation that continuous, long-term administration of low concentrations will produce potential responses of the respiratory system which may better reflect the mechanisms encountered on the roadside of freeways, rather than a short-term exposure to massive concentrations with large recovery periods between individual exposures.

Third, based on earlier findings of the variability of the physical properties of diesel particles with engine type [2,3] and studies of the effect of engine type and fuel by Huisingh, Bradow et al [4], it was felt that studies should concentrate primarily on the types of products which are expected to be used extensively on the roads. In this case, it was decided to test the emissions of a 1978 model production, 5.7 L, GM 350 diesel engine model burning Amoco 2D federal compliance fuel. This engine was an example of a GM light duty passenger car diesel at the time the experiment was conceived and begun, but may not be representative of current GM production or of other manufacturer's engines.

Fourth, the next important concern in the program is that the diesel engine be operated in a well-defined manner, since measurements by GM Research and others have shown that the particle's physical and chemical properties do vary with engine speed and load. Although it may be objected that realistic conditions may not be reflected by a steady state road-load condition as they are by a multi-mode operating cycle, it was felt that the former mode is more useful since it permits better definition of the quantity and quality of the received dose than a simulation of conditions on the road. Only a precisely defined dose will permit a thorough analysis of the biological response, and after a dose response relationship has been firmly established, the extrapolation to the real-life roadside concentration can be made.

Finally, reports of the high coagulation rate for diesel particles by Dolan et al [5] and in-house measurements of the variability in the chemical nature of the particle with exhaust conditions reported at the 71st APCA Meeting [2], have demonstrated the need for aerosol characterization and monitoring throughout the study. The primary concern in this regard is that the particles in the exposure chambers remain in the appropriate size range for realistic respiratory system interaction, that they contain a realistic amount of adsorbed hydrocarbon coating and that discrete dose levels be maintained throughout the study. For this purpose, several aerosol measurement instruments were custom designed and used to monitor the chamber diesel aerosol during the study.

PROTOCOL

Animals The animals exposed in this experiment were juvenile male laboratory rats (*Rattus norvegicus*) Fischer 344 strain and juvenile male guinea pigs (*Cavia porcellus*) Hartley outbred albino strain obtained from Charles River Breeding Laboratories. At arrival, both populations were approximately 5 weeks of age. Both species were quarantined two weeks before testing began. All animals were individually housed in wire inhalation caging, fed a balanced diet, and had food and water available *ad libitum*. Animals were rotated to equalize the average exposure and antibiotic impregnated cageboards were used to separate layers of cages and to reduce ammonia formation within the chamber.

The animals were observed daily and unhealthy individuals were reported for further evaluation. No animal was given chemotherapeutic treatment during the experiment. All animals which entered the experiment were accounted for, whether they were removed because of illness or culled for an evaluation of a biological response.

Caging and Chambers The cages were welded stainless steel mesh with removable feeders (Figure 1). Each could house six rats or three guinea pigs individually separated. Each stainless steel rack held 24 cages in 6 levels and provided automatic watering spigots. The four large exposure chambers (Figure 2) were constructed of stainless steel and glass. The chamber volume was 12.6 m^3 including the top and bottom cones. Each chamber held two racks (Figure 3) one containing 144 rats and the other 75 guinea pigs when filled.

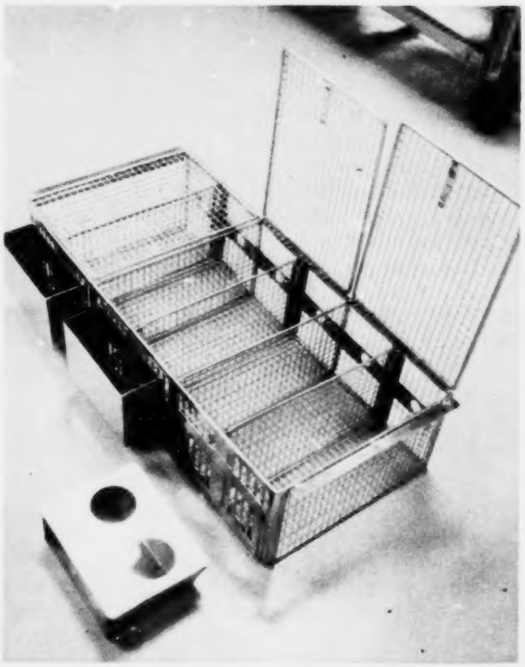

Figure 1 Stainless steel animal inhalation caging used in the chambers has feeders in the front and water spigot inlets at the back. Each module individually houses six rats or three guinea pigs (with dividers removed) and hangs suspended from tracks in a mobile cage rack.

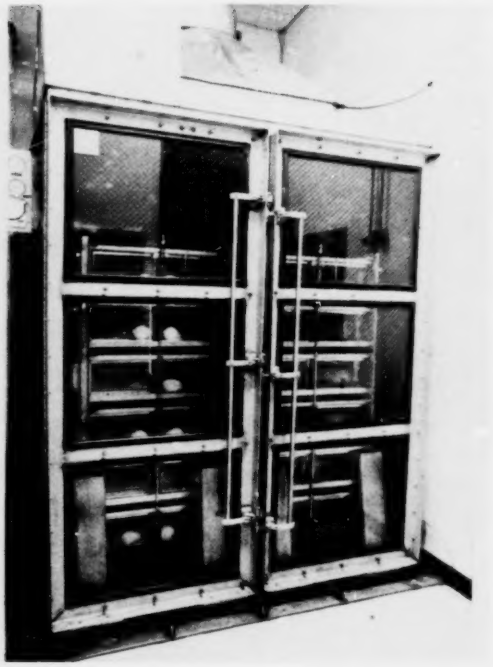

Figure 2 Frontal view of one 12.6 m^3 chronic inhalation chamber showing the air inlet section at the top, animal cages and cage racks with feeders, and internal automatic water supply. The entry track ramps are visible at the bottom of the chambers.

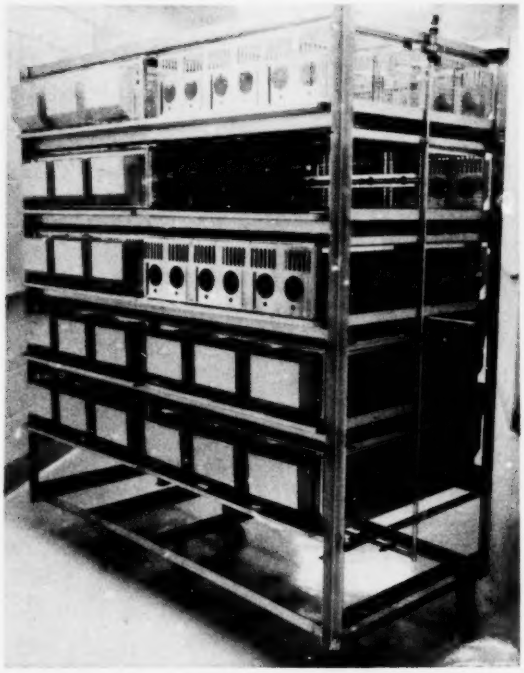

Figure 3 Stainless steel mobile cage rack showing cages in various states of assembly installed in the rack. The automatic watering system manifold and catch trays are also visible.

Particle Concentrations The nominal particle mass concentrations at the four exposure levels were 0, 250, 750 and 1500 $\mu g/m^3$.

Exposure Schedule Animals were exposed for 20 hrs/day, 5-1/2 days/week, for a total of 110.5 hrs/week (Figure 4). The largest nominal weekly exposure dose (Table 1) was 165,750 $\mu g/m^3 \cdot hrs$ at a concentration of 1500 $\mu g/m^3$, giving a calculated dose per animal of 60 $\mu g/m^3$.

The exposure was begun on Friday, December 8, 1978 and has continued without major interruption or mechanical breakdown. During the first two quarters of the experiment, two chambers were run at 250 $\mu g/m^3$ and none at 750 $\mu g/m^3$. One chamber was run at 750 $\mu g/m^3$ starting on June 8, 1979. Starting in the third quarter of the experiment, the exposure system ran unattended through the night.

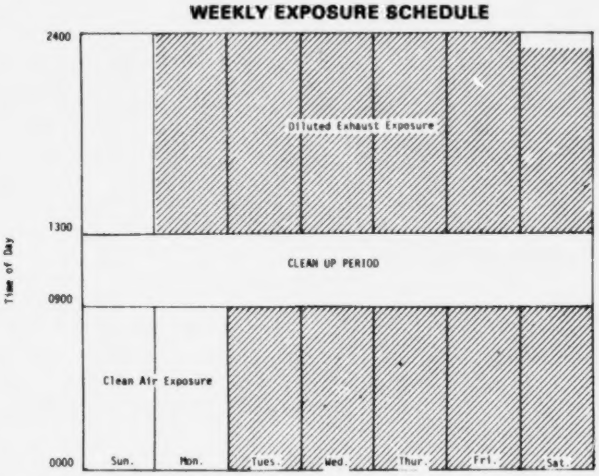

Figure 4 Weekly exposure schedule of 20 hours per day, 5-1/2 days per week for a total of 110.5 hours of exposure per week which was used in this study.

Table 1

Nominal Chamber Particle Mass Concentrations, Weekly Exposures, and Approximate Weekly Inhaled Dose/Rat

Diesel Aerosol Mass Concentration ($\mu g/m^3$)	Weekly Exposure Dose ($\mu g/m^3 \cdot hrs$)	Calculated Weekly Deposited Dose/Rat* (μg)
250	27,625	100
750	82,875	300
1500	165,750	600

* Assuming a minute volume of 200 mL and a total deposition efficiency of 30% of the inhaled dose.

Air Supply The exposure chambers were supplied with building air which was cooled to allow temperature and humidity control (Figure 5), prefiltered, passed through a commercial air purification unit containing potassium permanganate and activated carbon, split into separate flows for the individual chambers, reheated to the required temperature, HEPA filtered, drawn through the chamber, and exhausted through a fan on the roof which served all four chambers. The chambers were kept under 2 to 3 cm of water negative pressure. The total air flow through each chamber was 2.83 m^3/min which resulted in 13 air changes/hour.

The acceptable ranges of chamber temperature and relative humidity were 22 ± 2°C and 50 ± 20%, respectively. Each chamber was equipped with air flow and temperature alarms which could prompt security personnel to immediately seek instructions from a responsible person in the department during unattended operation.

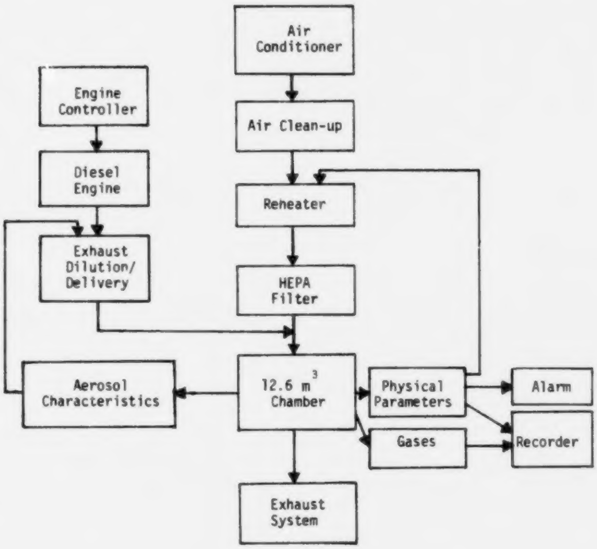

Figure 5 Flow diagram of the diesel exhaust exposure experiment.

Engine A production 5.7 L, four cycle indirect injection diesel engine built by Oldsmobile was run at a steady speed and load (1,350 rpm, 96 N·m) simulating a 65 km/hr cruise by a full-size passenger car as described in Figure 6. An electronic control circuit maintained the required settings during unattended operation. Amoco Type 2D federal compliance fuel (Table II) and Amoco 200 30W lubricating oil were used throughout the experiment.

ENGINE CHARACTERISTICS

ENGINE	1978 GM 350 DIESEL
DISPLACEMENT	5.7 L (350 IN.^{3})
COMPRESSION RATIO	22.5:1
BORE/STROKE	10.30/8.60 (4.057/3.385)
OPERATION CONDITION	65 KM/HR. ROAD LOAD (40.4 MPH)
SPEED	1350 RPM
TORQUE	96 N·M (71 FT. LB)
A/F RATIO (MASS)	56.2
VOLUMETRIC EFFICIENCY	0.832

Figure 6 Characteristics of the GM 1978 350D engine and its performance at this speed and load condition.

Exhaust Delivery To simulate passenger car emissions, the exhaust was drawn from a manifold located downstream of the muffler at a point where the end of a normal exhaust system would be (Figure 7). It passed through a valve assembly for adjusting the flow rate and into stainless steel delivery lines. These lines were heated to a temperature of 100 ± 15° C to keep the exhaust at tailpipe temperatures until it was rapidly diluted with clean air near the top of the chamber. The residence time of exhaust in a delivery line was estimated to be between 3 sec and 15 sec, depending on the chamber.

Table II

Analysis of AMOCO Type 2D Diesel Fuel
Combusted in Exposure Study

API Gravity @ 15.6°C	35.36
Specific Gravity @ 15.6°C	0.848
Lead, g/L	0.002
Sulfur, %	0.27
Fluorescent Indicator Absorption*, Vol. %	
Paraffins	65
Olefins	2
Aromatics	33
H/C, atomic ratio	1.77
Flash Point, °C	79.4
Viscosity @ 40°C, cSt	2.31
Cetane No.	44.3
Distillation	
IBP, °C	179.0
10%	212.5
50%	255.5
90%	306.5
FBP	335.0

*ASTM Method D1319

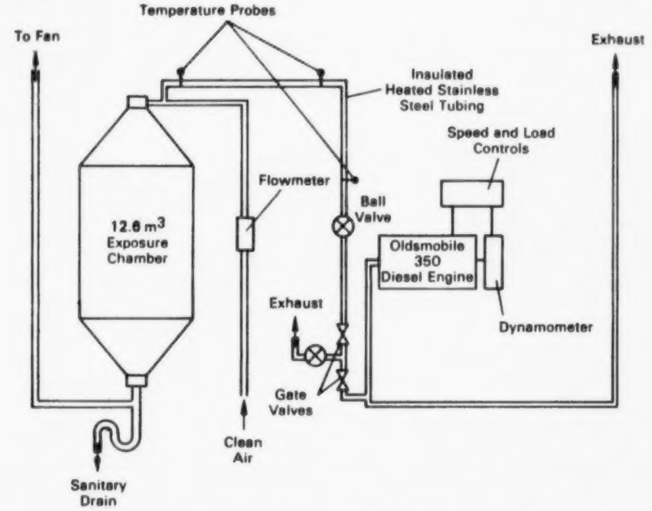

Figure 7 Engine exhaust delivery system showing the flow path through heated delivery lines from the engine to the exposure chambers. For simplicity, only one of the chambers is shown.

ROUTINE MONITORING

Methods The average aerosol mass concentration in each chamber containing diesel exhaust was determined from three filter samples (four filter samples during the first two quarters) which were periodically drawn during the exposure day. The filters were Pallflex T60A20 teflon coated glass fiber mat size 47 mm.

The size of the aerosol particles delivered to the chambers was measured routinely. A seven-stage cascade impactor was designed and used on a weekly basis to characterize the mass median aerodynamic diameter (MMAD) of the aerosol in the chambers and the percentage of the aerosol mass associated with particles less than 1.0 μm. A diffusion battery was also developed to measure the mechanical mobility diameter of the chamber diesel exhaust aerosol [6].

CHARACTERIZATIONS OF THE CHAMBER ENVIRONMENT

Particulate Uniformity Before the exposure started, the spatial uniformity of the particulate mass concentration was measured. Four open face 25 mm filters were drawn simultaneously at different locations throughout the chamber with the cages, racks, sanitary papers, feeders and animals in place. Several sets of four filters each were drawn on each of several days.

Typical variations in particle mass concentration at different locations in the chamber were 10% of the mean concentration. This variation includes measurements in the racks near the animals' breathing zones, as well as above and below the racks.

Clean Chamber Aerosol Another set of measurements probed the aerosol in the "clean" chamber using one cascade impactor placed above the cage rack and another in a cage from which the animal had been removed. The results are shown in Figure 8. In this set of measurements, the mass concentration of particles smaller than 1 μm is only about 10 $\mu g/m^3$. For both samples, the one in the cage and the one above, one-half of the aerosol mass is in very large particles (larger than 8 μm). The higher concentration in the cage is believed to be due to food dust and animal dander from nearby animals. This shows that most of the background aerosol in the clean chamber is much larger than the diesel particles and probably originates from activity of the animals.

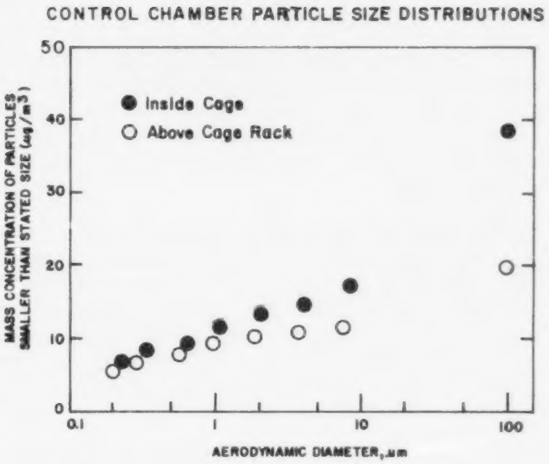

Figure 8 Control chamber particle size distribution showing that only about 10 µg/m³ aerosol mass is associated with particles smaller than one micrometer.

The gaseous components of the four inhalation atmospheres were characterized by a chamber air monitoring system (Figure 9). It consisted of a console of instruments to determine CO, NO_X, S, hydrocarbon, and ozone concentrations, as well as the dew point. The system included a General Automation GA 16/440 computer for process control functions, data logging, and data analysis. Measurements of CO and NO_X were begun in the second quarter and the remainder of the system became operational in the fourth quarter.

The gas analyzers were all maintained in accordance with the manufacturer's recommendations except where experience dictated more frequent service. Calibration mixtures of gases were checked against standards traceable to the National Bureau of Standards. Zero and span checks are made daily on all analyzers.

The bulk of the earlier gas concentration data was CO, NO, NO_2 and NO_X readings from the 1500 µg/m³ exposure chamber. This information was recorded continuously with a multiplexed strip chart recorder. Weekly gas samples were also taken for shorter periods of time in the other chambers.

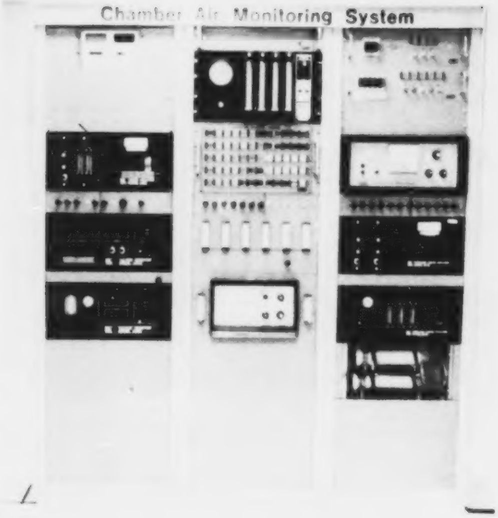

Figure 9 The chamber air monitoring system console containing instruments to monitor CO, NO_x, NO, NO_2, S, hydrocarbons, ozone, and dew point. Span, zero, and sample gases may be routed by manual or computer control.

Results The chamber aerosol mass concentration data for the four chambers was analyzed to give weekly concentration values as means and standard deviations for the first forty-one weeks of the experiment. As shown in Figure 10 the standard deviation of the concentration measurements did not exceed ± 30% of the mean weekly value. Through the first forty-one weeks of operation, the average concentrations in the three chambers were 254 ± 75, 759 ± 235 and 1519 ± 294 $\mu g/m^3$.

The diesel aerosol mass concentrations, depicted in Figure 10 by their weekly average values, had a variability of approximately 30% in each chamber regardless of whether control corrections were made hourly, as in the first fourteen weeks, or daily as was done thereafter. This variability was due both to the nonavailability of a fast response instrument for measuring chamber aerosol concentration for automatic closed loop control and the long time constant inherent in controlling chambers of this size. Despite these limitations, the four exposed animal populations did receive four discrete dose levels which had yearly

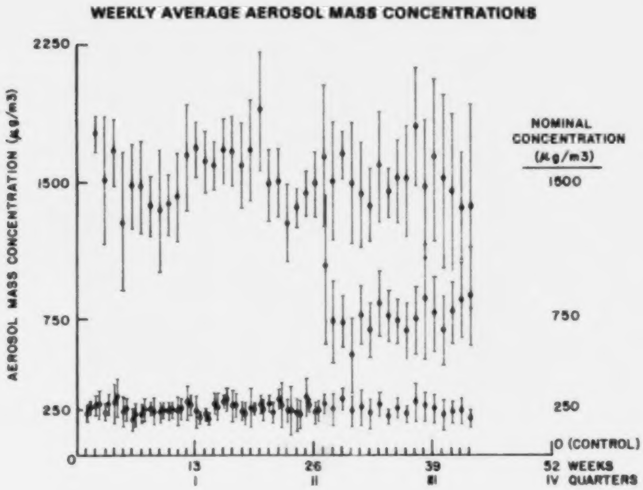

Figure 10 Weekly average diesel aerosol mass concentrations in the four exposure chambers determined from filter weight gains.

average concentrations within two percent of the target values. Throughout the experiment, attention was given to the size of the aerosol delivered to the chambers. Reports by many investigators have shown that diesel exhaust particles are released into the atmosphere in the submicron size range. Since there is little, if any, growth of this size of particle in the atmosphere, the particle has a high probability of depositing in the rat's deep lung regions, while significantly larger particles will not [7,8,9]. It was, therefore, a major design parameter of this experiment to ensure that the particles delivered to the chamber should arrive as a submicron aerosol and in the same size range as is released at the tailpipe. The cascade impactor measurements (Figure 11) have shown that the mass median aerodynamic diameter has been 0.2 μm with 90% of the mass associated with particles smaller than 1.0 μm in the three diesel exposure chambers throughout the experiment. These measurements were compared to electrical aerosol analyzer measurements which also determined the diesel particulate aerosol's mean diameter in the chamber to be approximately 0.2 μm.

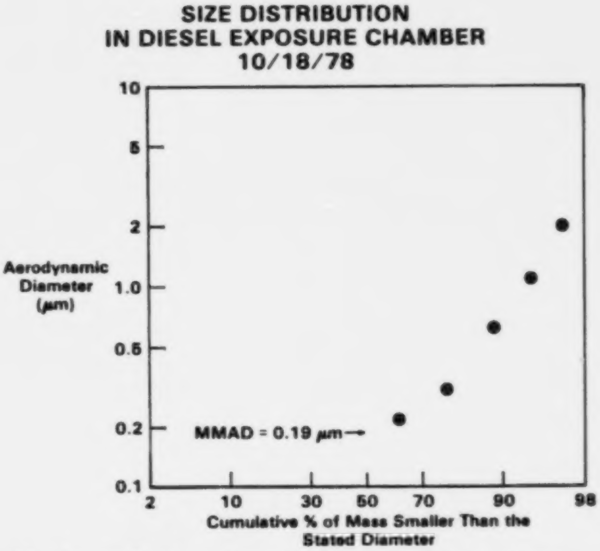

Figure 11 Aerosol size distribution of dilute diesel exhaust in exposure chamber showing a mass median aerodynamic diameter (MMAD) of 0.19 micrometer.

The means and standard deviations of the gas measurements made once per week are presented in the next two figures. All gas concentrations are expressed as mg/m^3 at our standard operating conditions of 22°C and 744 mm Hg.* The gas analysis data in these slides is the result of weekly samples analyzed over shorter periods of time than the filter mass readings, and therefore is not as heavily time weighted. The carbon monoxide concentrations, corrected for ambient levels (Figure 12), generally followed the trend of the diesel particle mass concentrations over the period of time monitored. Discrepancies at specific week's readings appear to result from the shorter averaging times in the CO measurements rather than a consistent difference between the ratio of aerosol to CO concentration. The same applies to NO_x concentrations measured during weekly analysis (Figure 13) and based on a measured average ratio of 94% NO to 6% NO_2 in the chamber. With the exception of the dip at the mid-year which was caused by a change of engines after 267,000 km of

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*Mass concentrations of gases at our standard operating conditions of 22°C and 744 mm Hg are calculated from

$$C\ (\mathrm{mg/m^3}) = \frac{\text{Molecular Weight}}{24.73} \times C\ (\mathrm{ppm})$$

operation, NO_x also followed the chamber particle mass concentration. The CO and NO_x concentrations and the temperature and relative humidity recorded at the time of the weekly measurements are summarized with their means and standard deviations in Table III.

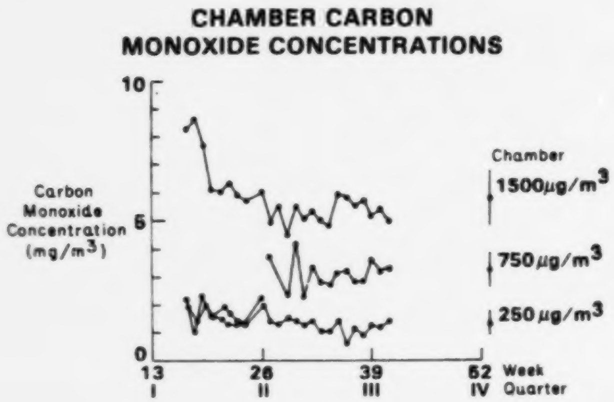

Figure 12 Carbon monoxide concentrations measured during weekly sampling of chambers and corrected for ambient concentrations.

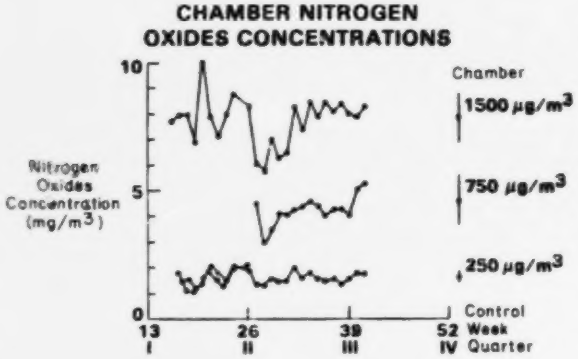

Figure 13 Nitrogen oxide concentrations measured during weekly sampling of chambers.

Table III

Inhalation Chamber Environmental Parameters*

	Chamber #1 Control	Chamber #2 1500 $\mu g/m^3$	Chamber #3 750 $\mu g/m^3$	Chamber #4 250 $\mu g/m^3$
Chamber Temp.	20.8± 0.9°C	21.7 ± 0.8°C	21.4 ± 1.0°C	21.7 ± 0.8°C
Relative Humidity	56 ± 6 %	58 ± 7%	56 ± 6%	55 ± 7%
Dilution Ratio (approx.)	-	40:1	80:1	240:1
Nitrogen Oxides	0.0 mg/m^3	7.9 ± 1.0 mg/m^3	4.7 ± 1.0 mg/m^3	1.7 ± 0.2 mg/m^3
Carbon Monoxide	1.6± 0.6 mg/m^3	7.5 ± 1.0 mg/m^3	4.8 ± 0.8 mg/m^3	2.9 ± 0.7 mg/m^3
Carbon Monoxide (corrected for ambient)	0.0	5.8 ± 1.0 mg/m^3	3.2 ± 0.6 mg/m^3	1.3 ± 0.4 mg/m^3
Aerosol Concentration	11 ± 6 $\mu g/m^3$	1519 ± 294 $\mu g/m^3$	759 ± 235 $\mu g/m^3$	254 ± 75 $\mu g/m^3$

* Information for chambers 1, 2, and 4 are for the period 4/3/79 to 10/11/79. Information for chamber 3 is from the period 6/13/79 to 10/11/79.

In summary, dilute diesel exhaust from a 1978 GM 5.7 L 350D engine was continuously delivered to three of these chambers for 20 hours per day, 5-1/2 days per week at concentrations of 250, 750 and 1500 $\mu g/m^3$, while the fourth chamber received clean air. After 41 weeks of exposure, the average diesel particulate concentrations of 254 ± 75, 759 ± 235 and 1,519 ± 294 $\mu g/m^3$ are all within 2% of the target values. In addition, the aerosol delivered to the chambers has been shown to be similar in size to diesel exhaust particles released into the atmosphere. Chronically exposed animals from this experiment have been supplied to intramural and extramural investigators through the first exposure year which has concluded the first week of December, 1979.

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General Discussion

J. PATTISON: What was the ratio of NO to NO_2 fractions in the high chamber?

R. SCHRECK: We were running about seven NO_x levels, and the NO_2 to NO ratio, which I reported for that chamber, was running about 94 percent NO to about six percent NO_2 at that operating location. That is done with the engine operating, but we are running a steady mode so we can relate that back. It is one of the problems with running a dynamic cycle.

P. MOGAN: Do you have an SO_2 level approximate?

R. SCHRECK: I don't have an SO_2 level. We did some computations with sulphur based on fuel sulphur, and that is all we have to date. The SO_2 instrument required some safety-related things which precluded our getting it operational in time to make a significant number of measurements for this meeting. Based on the calculations of the amount of fuel going through the system, we had about 60 milligrams of sulphur per cubic meter.

D. HOFFMANN: Were your five days a week exposure for logistics reasons?

R. SCHRECK: Yes.

D. HOFFMANN One may argue that you don't live only from Monday to Friday in gasoline air - diesel polluted air - but you also live Saturday and Sunday. Is it logistic reasons? My next question is did you ever make any effort to look for volatile nitrosamines?

R. SCHRECK: We are not analyzing for nitrosamines in diluted air. As far as logistic reasons, yes, in fact, at the time we went fully automated, we could have run seven days a week. We didn't want to interrupt the procedure, since we had already set out a protocol and had already gone several quarters into the protocol. We could have gotten a little higher dose per week but we thought it would be better to go with the pattern that had already been established.

PHYSICAL CHARACTERIZATION OF DIESEL EXHAUST PARTICLES IN EXPOSURE CHAMBERS

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ABSTRACT

Since the deposition of particulate in the respiratory system is strongly influenced by particle size, a correct assessment of this parameter is important for any inhalation experiment studying the potential health effects of air pollutants. Measuring the distribution of particles according to their aerodynamic diameter and mechanical mobility diameter is crucial in analyzing the deposition of submicron particles in the lower respiratory system.

Cascade impactor measurements of diluted diesel exhaust in 12.6 m^3 animal exposure chambers in the GMR Biomedical Science Department showed that the mass median aerodynamic diameter of the aerosol was 0.2 μm with 88% of the mass in particles smaller than 1 μm. Diffusion battery measurements showed that the mass median mechanical mobility diameter was about 0.11 μm.

Transmission electron micrographs of particles deposited on chamber surfaces revealed both agglomerates and nearly spherical particles. The particles in these chambers are similar in size and shape to diesel particles described elsewhere.

The flux of diesel particles to food surfaces was measured. Calculations of the expected daily dose by inhalation and by feeding showed that the "worst case" dose by feeding was only about one-tenth the dose by breathing.

INTRODUCTION

This paper covers three types of physical characterizations of the dilute diesel exhaust aerosol in the GMR Biomedical Science Department's 12.6 m^3 animal exposure chambers. Such characterizations are necessary to define the exposure conditions. The three topics are: I. Health Effects Relevant Sizing, II. Transmission Electron Microscopy, and III. Particulate Doses by Feeding and Breathing.

I. Health Effects Relevant Sizing of the Diesel Aerosol in Exposure Chambers

The objective of this work was to routinely measure the size distribution of the diesel exhaust aerosol in the GMR Biomedical Science Department's inhalation exposure chambers. This was necessary to assure the animals were given diesel exhaust particles of realistic size and to provide information relevant to deposited dose. There are many techniques available for obtaining size information. We chose two techniques which measure the distribution of particulate mass versus aerodynamic diameter and mechanical mobility diameter. These techniques are especially useful in an inhalation experiment because lung deposition depends mainly on aerodynamic diameter for particles larger than about 0.3 μm and on mechanical mobility diameter for smaller particles. Knowing the distributions of particulate mass versus these two size parameters allows the deposited dose to be estimated directly. The calculation of deposited dose using any other size information requires extrapolations which have a high uncertainty, especially for irregularly-shaped particles such as those in diesel engine exhaust.

We used a cascade impactor to measure the distribution of particulate mass versus aerodynamic diameter. Successively smaller particles are collected on each stage of an impactor as the air cascades through a series of jets. The construction of our impactors followed that of Mercer [1], but the number and size of the jets in each stage was changed. This allowed a smallest cutoff aerodynamic diameter of 0.23 μm while sampling at 4 L/min. The collected particles experienced a pressure reduction of less than 15 kPa. Commercially available impactors had too large a cutoff diameter causing most of the diesel particles to be collected on the back-up filter, too low a flow rate requiring long sampling times, or too high a pressure reduction favoring evaporation of volatile components.

The mass deposited on the glass collection plate in each stage and on the back-up filter was measured with an electrobalance. The raw data were analyzed assuming an ideal collection efficiency for each stage and plotting the data on log-probability graph paper [2]. The cutoff diameter was calculated from information in the literature on the calibration of similar impactors [3].

A typical result for the chambers containing diesel exhaust particles is shown in Fig. 1. The percent of mass on particles smaller than each cutoff diameter is plotted against the cutoff diameters on logarithmic probability paper. If the points fell on a straight line, the distribution would be log normal. The parameters reported for each distribution are: 1) the mass median aerodynamic diameter (MMAD), and 2) the percent of mass on particles smaller than 1.0 μm (PC1). The result of 54 measurements in all the chambers containing diesel exhaust was MMAD = 0.20 (±0.03) μm and PC1 = 88 (±5)%. The uncertainties represent one standard deviation.

Two points should be emphasized regarding this sizing technique: 1) slight revision of the reported size may be necessary after a full calibration of our impactors is completed, and 2) the multi-jet design had larger wall losses on the lower stages than expected (up to 20% of the total mass sampled), but correcting for this loss seems to change the mass median aerodynamic diameter by only about 10%.

Because 50-60% of the aerosol mass was caught on the backup filter of the impactor, it was necessary to size the aerosol by a different technique--one which is sensitive to smaller particles. The principle of the diffusion battery is that as air is pulled through a channel particles near a wall may hit it due to their Brownian motion and stick. The concentration of aerosol upstream and downstream of the channel was measured and the fraction penetrating the channel was related to the aerosol's distribution vs. diffusion coefficient.

The diffusion battery consists of several diffusion cells each of which is a matrix of channels. Some of the cells are equivalent to a single channel 4.6 km long.

Fig. 2 shows the data of 18 runs combined. The fraction of mass concentration penetrating the cell is plotted against the collection efficiency parameter of that cell. The collection efficiency parameter is a combination of the effective length of the cell and the volume flow rate of air passing through it. The line is the theoretical penetration curve for an aerosol of mass median mechanical mobility diameter = 0.11 μm. The width of the distribution is specified by $\sigma_g \sim 4.5$. This data analysis was done

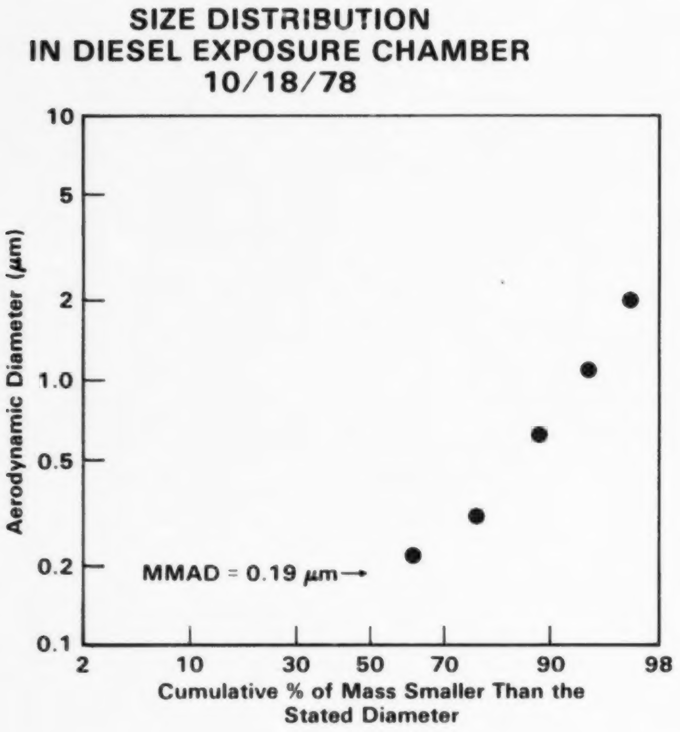

Fig. 1 - Typical log-probability plot of the size distribution of diesel particles in the exposure chambers as measured with a cascade impactor.

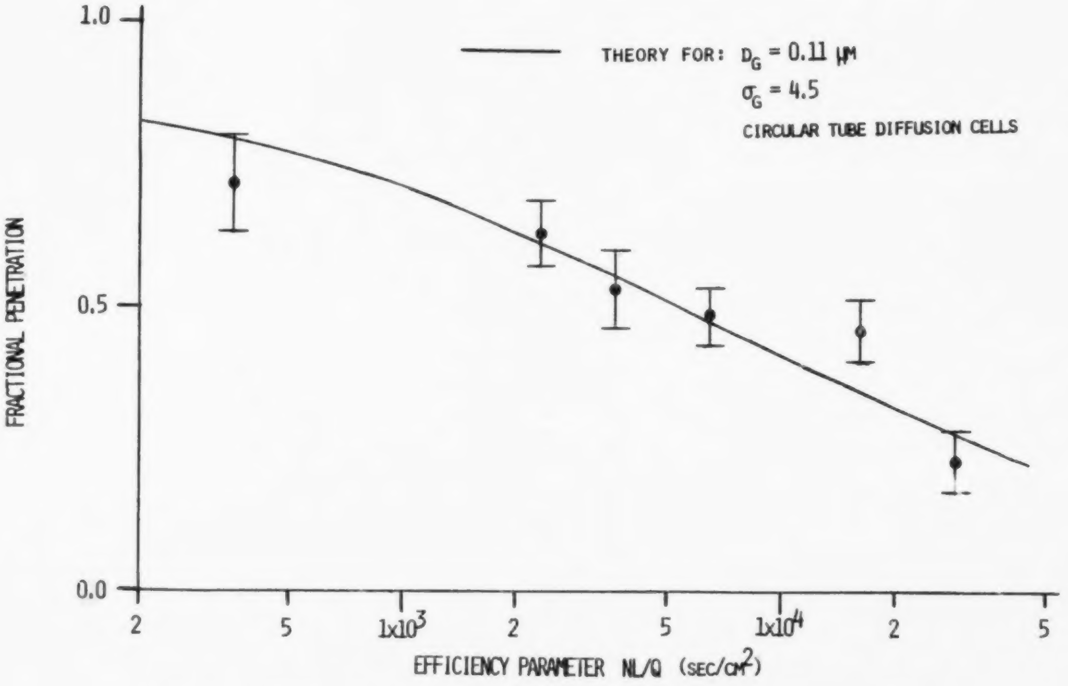

Fig 2 - Analysis of diffusion battery data which is a summary of 18 samples from all three exposure chambers.

using the theory appropriate to channels of circular cross-section [4], whereas our channels were triangular. The difference should not be large. The results may have to be revised slightly once a full calibration is performed.

II. Transmission Electron Microscopy

Many electron micrographs of diesel particles have been published [5,6]. Most show agglomerates roughly 0.1-0.3 μm in size with some smaller singlets.

Fig. 3 shows particles which were collected from diluted engine exhaust using a TSI Model 3100 electrostatic sampler. Many chain and cluster agglomerates are seen. The pictures in Fig. 4 were taken from grids which had been attached to the chamber walls overnight. Note that many more small singlet particles are seen due to their preferential deposition by diffusion. Fig. 5 shows some agglomerates and unusual particles which were found in the chambers. The micrographs in Figs. 3 and 4 were taken at random on the specimen grids, but the micrographs in Fig. 5 were not.

The conclusion drawn from these micrographs is that the diesel exhaust particles in our chambers are similar in size and shape to those previously reported.

III. Dose by Feeding and Breathing

In a whole body exposure to diesel aerosols, the animals' intake of particulate occurs through feeding and grooming as well as through breathing. The dose received by all routes should be known to help understand possible causes of any biological effects which may be seen. The objective of this work was to estimate and compare the doses by feeding and breathing to the animals in the GMR Biomedical Science Department's diesel exposure experiment. The dose by grooming was not estimated in this study.

An experiment was performed to determine the mass flux of diesel particulate to food surfaces in this exposure. Transmission electron microscope specimen grids were placed on food surfaces in the exposure chamber overnight. The deposit was analyzed by transmission electron microscopy to obtain the volume surface density S_v of deposited particles:

$$S_v = 1.6 \times 10^{-8} \frac{\text{cm}^3 \text{ particles}}{\text{cm}^2 \text{ surface}}$$

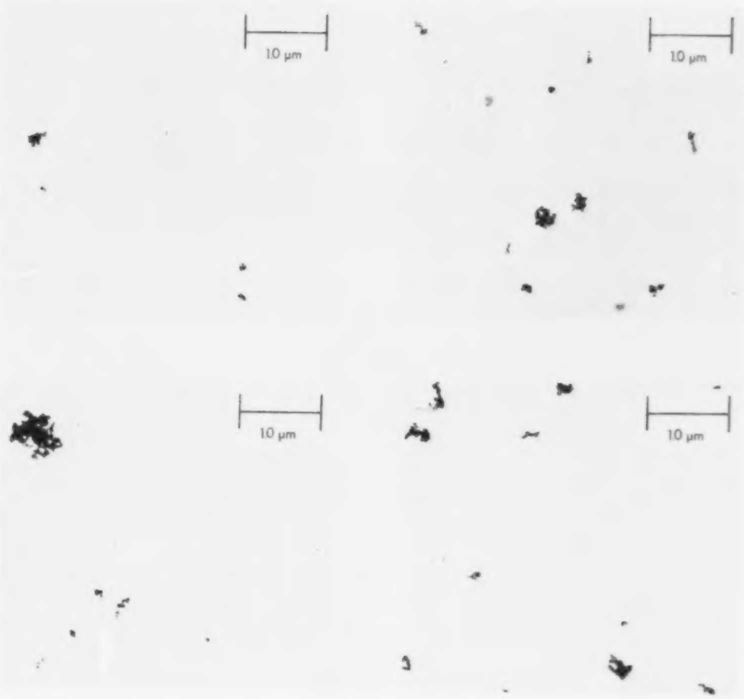

Fig. 3 - TEM micrographs of randomly chosen diesel exhaust particles collected from the tailpipe using an electrostatic sampler.

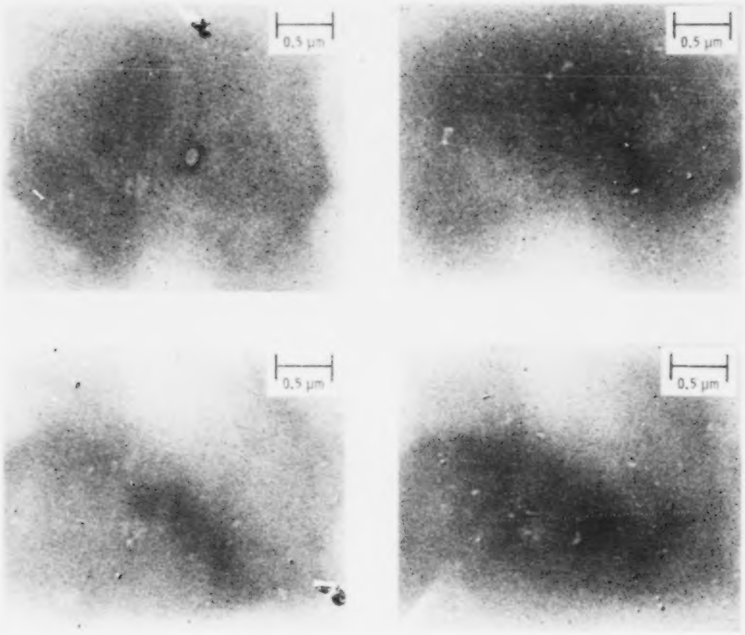

Fig. 4 - TEM micrographs of randomly chosen diesel exhaust particles collected by placing a specimen grid on the exposure chamber wall overnight.

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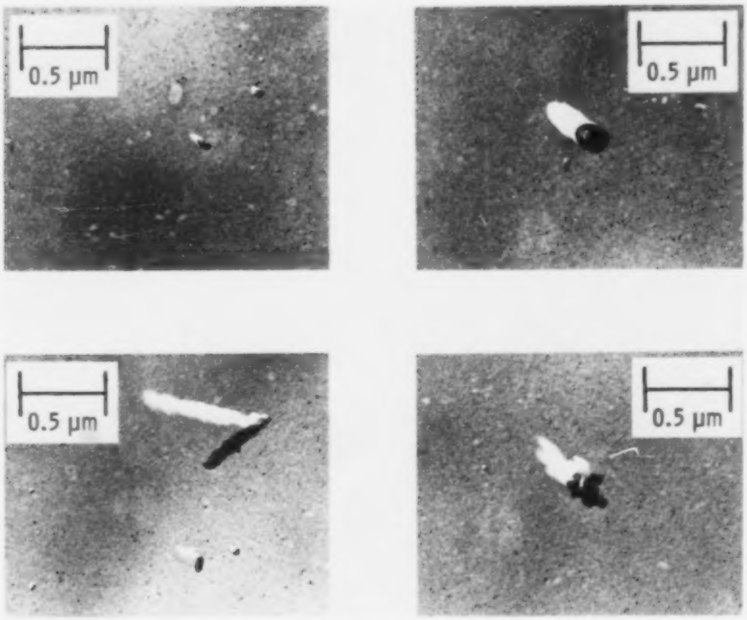

Fig. 5 - TEM micrographs of selected diesel exhaust particles collected by placing a specimen grid on the exposure chamber wall overnight.

The equivalent volume diameter of each particle was estimated using a ruler. I estimated the uncertainty in S_v to be about a factor of two due to the crude method available for estimating particle volumes of agglomerates from electron micrographs.

The mass flux J of diesel particulate to food surfaces was calculated to be:

$$J = 1.4 \times 10^{-5} \frac{m}{s} \cdot C$$

where C is the mass concentration of airborne diesel particles. The following information was needed to calculate the mass flux: 1) the volume surface density of deposited particulate S_v, 2) an estimate of the density of diesel particles ($\rho p = 1.5$ gm/cm^3), 3) the time H that the grid was exposed to diesel particles in the chamber, 4) the mass concentration during that exposure C_{Exp}, and 5) the fact that the mass flux should be proportional to the mass concentration of airborne particles. The mass flux may be different for different size particles, feeder configurations, or air flows.

The dose by feeding was estimated using the formula:

$$D_F = J \; T \; A.$$

The length of an exposure day T was 20 hours. The area of food surface which the animals kept clean of particles was A. Estimating this area involved large uncertainty. The total area of food surface available for diesel particle deposition was estimated to be 30 cm^2 for the rats and 100 cm^2 for the guinea pigs. I used these values for A. This was equivalent to making the "worst case" assumption that the animals ate from all the available area of food keeping it clean of particles. This calculation gave:

$$D_F = 3 \frac{\mu g \;\; m^3}{day \;\; mg} \cdot C \text{ for the rats}$$

$$D_F = 10 \frac{\mu g \;\; m^3}{day \;\; mg} \cdot C \text{ for the guinea pigs.}$$

If the animals ate from only one part of the food surface, the true dose by feeding would be much lower than this "worst case" estimate.

The dose by breathing was estimated using the following formula:

$$D_B = V_m \; T \; C \; E.$$

The animal's minute volume V_m is the volume of air inhaled in a minute. The length of an exposure day T was 20 hours, the mass concentration of airborne particles was C, and the deposition efficiency was E.

The available minute volume data [7-10] for rats and guinea pigs is shown in Figure 6. Also shown are two correlations between body mass and minute volume based on data from a number of species. From the graph, I estimated the minute volume of the 400 g rats in this experiment to be 200 ± 50 cm^3 and that of the 1000 g guinea pigs to be 250 ± 100 cm^3. There was little data available for guinea pigs and it did not agree with the general correlation, so extrapolation to the minute volume of the 1000 g guinea pigs carried larger uncertainty than the minute volume estimation for the rats.

The deposition efficiency E was taken to be 0.3 based on total deposition (head, stomach, lungs, etc.) seen by Raabe, *et al* [10] in experiments with rats and hamsters using an aerosol which was similar in size to diesel particles. The deposition efficiency was the same for the two rodent species, so I applied it to the guinea pigs as well as to the rats.

This calculation gave:

$$D_B = 72 \frac{\mu g \;\; m^3}{day \;\; mg} \cdot C \text{ for the rats}$$

$$D_B = 90 \frac{\mu g \;\; m^3}{day \;\; mg} \cdot C \text{ for the guinea pigs.}$$

The comparison of the doses by feeding and breathing in Table I shows that the "worst case" estimate of the dose by feeding is only about one-tenth of the dose by breathing for the animals in the GMR Biomedical Science Department's diesel exposure experiment.

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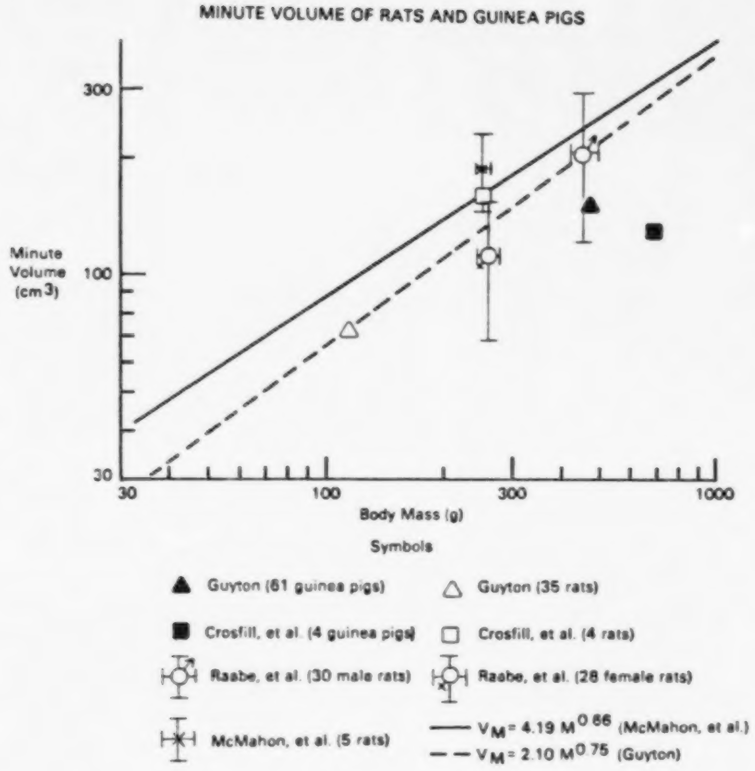

Fig. 6 - Minute volume of rats and guinea pigs from the literature.

TABLE 1
RELATIVE DAILY DOSE*

	BREATHING	EATING
RATS	72	3
GUINEA PIGS	90	10

*MULTIPLY BY DIESEL PARTICULATE MASS CONCENTRATION IN MG/M^3 TO OBTAIN DOSE IN μG/DAY.

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General Discussion

G. COOPER: In the previous paper it was stated that the exhaust was kept at 100°C, mainly, I guess, to make sure that the particles were the same size as came out of the exhaust. Is that correct?

S. SODERHOLM: I think the main concern in keeping the particles at 100°C is to freeze them at the end of the tailpipe until they reach the top of the chamber where they could be rapidly diluted. I would say the main consideration in keeping the size constant is to keep the residence time between the tailpipe and the chamber as low as possible. In our case it was less than 15 seconds.

G. COOPER: Do you have any information in terms of particle sizes by the distance from the exhaust pipe?

S. SODERHOLM: No, we haven't actually measured that, and certainly it is an imperfect system in the sense that we cannot fully simulate what happens in the atmosphere. We haven't actually measured that, but our size measurements come out very similar to almost all others I have heard.

W. PETERS: Have you any information on whether the chemical composition of the extract on the aerosols remains invariant, number one, during that passage which you said had a residence time from about 10 seconds to a minute from the exhaust to the chamber and then while they are within the chamber?

S. SODERHOLM: The residence time was three seconds to 15 seconds - same order of magnitude. We don't actually have chemical data about extractabilities, but some Ames assay tests have been done.

B. AMES: All three chambers look alike for the first six months, so there is no difference between the three chambers we have studied so far.

R. MCCLELLAN: I was very pleased to see the data that you presented Dr. Soderholm. I think it addresses an important point, particularly the latter information on the quantitative assessments of the actual deposition. We have done somewhat similar studies in terms of attempting to assess the amount of material deposited in the lungs with this whole body type of exposure and the amount through the GI tract. Our data or preliminary assessment of it, would suggest that a greater amount of material is actually cleared through the GI tract as a result of grooming than is deposited and retained within the pulmonary region. In addition, using the material that you presented from the studies at our institute on deposition, or using our data, 30 percent of the inhaled material was deposited in the respiratory tract, and of that, two thirds was in the pulmonary region giving you 72 micrograms. Of course, from that same data, you know that the other one-third was cleared by the GI tract so it amounts to 36 micrograms there. The conclusion is, I think, pretty clear that more material is actually cleared through the GI tract in terms of mass per day than is actually deposited in the lungs.

S. SODERHOLM: I think that is a very good point. The GI tract sees more particulates passing through than the lungs, and we shouldn't forget that in any systemic effects we may see.

D. KITTLESON: Where did you take your samples for size measurement?

S. SODERHOLM: We took them from just above the cage rack, right in the chamber. We put the whole impactor in the chamber.

PULMONARY FUNCTION TESTING OF ANIMALS CHRONICALLY EXPOSED TO DILUTED DIESEL EXHAUST

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ABSTRACT

The purpose of this work was to assess the potential effect that chronic inhalation of diesel exhaust may have on lung mechanics and lung volumes. Noninvasive pulmonary function tests have been conducted repeatedly on 25 rats exposed to diesel exhaust at a particulate concentration of 1500 $\mu g/m^3$, for 20 hours/day, 5-1/2 days/week for 267 days. The same tests were conducted on 25 clean air control animals. When the data is normalized, there are no apparent functionally significant changes occurring in the lungs that may be attributed to the chronic inhalation of diesel exhaust.

INTRODUCTION AND BACKGROUND

The purpose of this research project was to determine if the chronic inhalation of diesel exhaust may affect the mechanics and volumes of the lungs. It is well known that the diesel engine emits particulate matter of a size that may be inhaled and retained by the lungs and thus has the potential for altering the structure and composition of this organ. Previous studies of population groups occupationally exposed to diesel exhaust, reported by Battigelli et al [1] in which workmen in railroad engine houses were studied, failed to show a clear lung function effect attributable to the diesel exhaust. This lack of effect was confirmed by two Swedish studies, those by Jorgensen and Svensson [2] and Attfield [3]. At a recent meeting of the National Research Council Diesel Impacts Study

Committee, Reger and Hancock reported a higher incidence of subjective symptoms and marginal decrements in several pulmonary function tests in mine workers exposed to diesel machinery exhaust underground [4]. However, no data on exposure to coal dust were presented and the possibility that the mines equipped with diesel machinery might have had higher concentrations of coal dust cannot be excluded. While some controlled animal studies have been conducted on the component gases of diesel exhaust and how they affect pulmonary function [5,6] and a few acute studies have been carried out using whole diesel exhaust [7,8], no controlled chronic studies involving whole diesel exhaust have been carried out. This study attempts to resolve the question of the possible effect of diesel exhaust inhalation on lung function by studying the effects of long term exposures to diluted diesel exhaust on lung mechanics and volumes in an animal model.

METHODS

The technique for noninvasive testing of pulmonary function in rodents as described by Mauderly et al [9] was utilized with slight modifications. We have found these methods suitable for consecutive tests on the same animals during the entire exposure period.

Male Fischer 344 laboratory rats (*Rattus norvegicus*), were anesthetized by placement in a chamber through which 5% halothane (Ayerst Laboratories, New York, N.Y.) in oxygen was continuously flowing. Once the animal was deeply anesthetized as evidenced by the rate and depth of breathing, he was removed from the chamber and the trachea was intubated with a special catheter with the aid of a laryngoscope. The catheter was modified from a Jelco Cathlon IV 14 gauge catheter placement unit. The animal was then placed in a plethysmograph as shown in Figure 1. The female luer end of the tracheal catheter was attached to the male luer end of a 'Y' fitting which projects through the plethysmograph wall into the chamber. The other ends of the Y fitting protruded from the chamber and terminated in female luers.

An anesthetic chamber was attached to one of the female luer fittings so that the animal could be maintained under anesthesia. The percentage of halothane in oxygen was regulated so that each animal spontaneously breathed 60 breaths per minute. The animal could thus be kept anesthetized and intubated in this manner for several hours.

Transpulmonary Pressure

Transpulmonary pressure (the pressure difference between

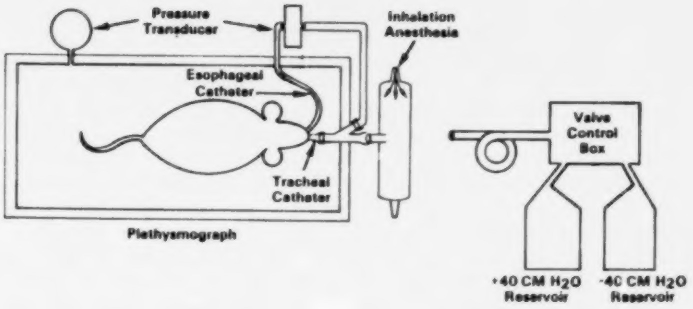

Figure 1 Schematic diagram of the noninvasive pulmonary testing preparation showing a rat placed in the plethysmograph.

the airway and pleural sides of the lungs) was measured continuously in the following manner. A fluid-filled catheter made from a premature infant (size 5 French) feeding tube, and attached at one end to a differential pressure transducer, was placed down the esophagus until it laid within the thoracic cavity and was therefore subjected to the same pressures as the pleural space. The pressures recorded in this manner were thus an indication of the pleural pressures. Another fluid-filled catheter was connected to the branch of the Y fitting that passes through the plethysmograph wall and thus monitors airway pressure. This catheter was attached to the other side of the differential transducer. The net output of the transducer (the transpulmonary pressure), was input to a Buxco Model 6 Pulmonary Function Monitor (Buxco Electronics, Sharon, CT).

Lung Airflow, Volume of Air Breathed and Respiratory Rate

The pressure changes within the plethysmograph were monitored via a transducer connected to the Buxco Pulmonary Function Monitor. The system was calibrated so that a given change in plethysmograph pressure equaled a given amount of air inhaled or exhaled by the animal. This pressure signal was differentiated by the Buxco monitor so that instantaneous air flow into or out of the lungs was also continuously recorded. The Buxco monitor also integrated the inspiratory peaks of the volume signal and supplied a digital readout of the respiratory rate which was updated every four seconds.

Fast Forced Expiratory Maneuver

In this maneuver the anesthetized animal was forced to inhale maximally and then exhale forcibly and maximally. The anesthesia chamber was removed from the tracheal intubation fitting and the animal hyperventilated by the use of a syringe for 10-15 seconds so that he became temporarily apneic. A section of tubing that terminated at one end in a male luer fitting was then quickly inserted into the female luer fitting that protrudes from the plethysmograph. The other end of this tubing was connected to a control box with solenoids that open and close the connections of this tube to either of two air reservoirs. One reservoir was maintained at +40 cm H_2O and the other at -40 cm H_2O. Once the tubing was in place, the line was opened to the +40 cm H_2O reservoir and the animal's lungs were forcibly inflated. When a transpulmonary pressure of approximately 30 cm H_2O was reached, a specially designed sensing circuit automatically closed the solenoid to the pressure reservoir, paused for .2 seconds and opened the solenoid to the vacuum reservoir. This procedure quickly withdrew the air from the animal's lungs. The rate limiting step in air flow was the resistance of the animal's airways and the stiffness of the lungs. During this maneuver the airflow, transpulmonary pressure and lung volume changes were taken from the Buxco monitor output and recorded on magnetic tape.

Functional Residual Capacity (FRC) Maneuver

In this maneuver the animal was hyperventilated and the tracheal catheter plugged so that no air could flow. As the animal tried to breathe, the chest wall and lungs were expanded. This consequently rarified the air within the airways and airway pressure decreased. These changes in lung volume and airway pressure were recorded. After approximately 8 seconds of the animal's attempting to breathe against what is essentially a closed-off airway, the plug was removed and the animal is allowed to breathe.

DATA ANALYSIS

Spontaneous Breathing During Anesthesia

As previously mentioned, during this phase of the experiment, the concentration of anesthesia was adjusted until a respiratory rate of approximately 60 breaths/sec was achieved. From the Buxco Monitor the following parameters were recorded: tidal volume, peak to peak transpulmonary pressure, peak inspiratory flow and peak expiratory flow. The tidal volume was divided by the peak to peak transpulmonary pressure, the result being dynamic compliance.

Forced Fast Expiratory Maneuver

The data from this maneuver which was stored on magnetic tape was played back and plotted on an X-Y recorder in the following manner. Volume of air exhaled was plotted on the X axis while the simultaneous air flow was plotted on the Y axis. This generates a typical "flow-volume" curve as displayed in Figure 2. The data extracted from this type of a curve were peak expiratory flow rate (PEFR), percent of vital capacity at PEFR, maximum expiratory flow rate at 50% of vital capacity (MEF_{50}), maximum expiratory flow rate at 40% of vital capacity (MEF_{40}), maximum expiratory flow rate at 20% of vital capacity (MEF_{20}), mean expiratory flow between 50 and 0% of vital capacity (Mean EF_{50-0}), and maximum expiratory flow rate at 25% of vital capacity minus the mean EF_{50-0} ([MEF_{25}]-[mean EF_{50-0}]).

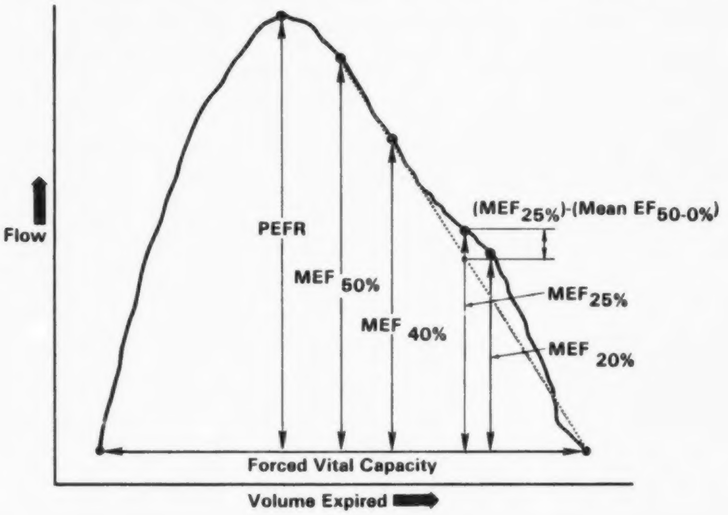

Figure 2 A typical flow-volume curve produced by the forced expiratory maneuver.

Figure 3 shows duplicate flow-volume curves obtained from two animals five minutes apart. They indicate that while the shape of the curve was fully reproducible for each animal, they did vary from animal to animal. On the average, there was less than 5% difference in the data calculated from the first and second flow-volume curves for each animal. In addition, no systematic difference was found between the data collected from the first and second curves for each animal.

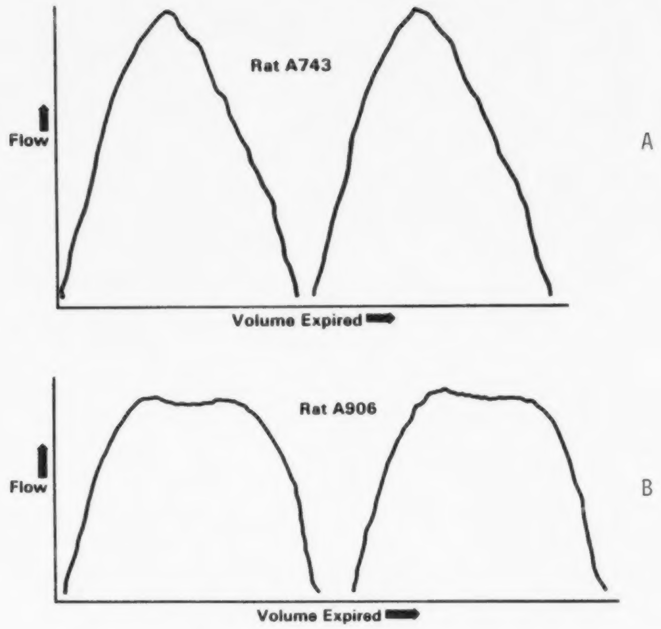

Figure 3 Duplicate flow-volume curves from two animals.

In addition to the flow-volume curve, a plot of volume inhaled and exhaled (Y axis) during the forced maneuver versus time (X axis) is plotted. From this volume-time plot the following values are derived: forced expiratory volume in 0.1 seconds (FEV$_{.1}$), forced vital capacity (FVC), FEV$_{.1}$/FVC(%), inspiratory capacity (IC) and expiratory reserve (ER). See Figure 4.

Typically, two forced fast expiratory maneuvers were performed on each animal and the mean of the two values used as the value for each animal.

Functional Residual Capacity (FRC)

By taking the airway pressure and volume changes recorded during the FRC maneuver and inserting them into the following equation which is derived from Boyle's Law, the functional residual capacity of the lungs was determined.

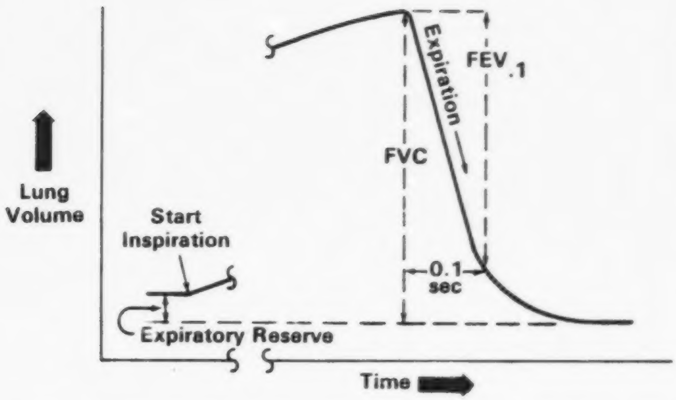

Figure 4 A typical volume-time curve produced by the forced expiratory maneuver.

FRC + instrument dead space =

$$[(P_B - P_{H_2O} - \Delta P_{ao}) \times \Delta V] / \Delta P_{ao}$$

where P_B = barometric pressure, P_{H_2O} = water vapor pressure at the animal's body temperature, ΔP_{ao} = change in airway pressure during inspiratory effort and ΔV = change in lung volume during inspiratory effort.

This maneuver was usually performed in duplicate and the mean of the two values used as the result for each animal.

Derived Lung Volumes

Given the values measured from the procedures and analyses mentioned above, several parameters were derived:

Inspiratory reserve volume [IRV] =
(inspiratory capacity) - (tidal volume)

Total lung capacity [TLC] =
(inspiratory capacity) + (functional residual capacity)

Residual volume [RV] =
(functional residual capacity) - (expiratory reserve volume)

These derived lung volumes were calculated twice for each animal and the mean used as the value for that animal.

Normalization of Data

The body weight of the two animal groups, as well as the percent increase in body weight during the exposure period, are displayed in Figures 5A and 5B. To compensate for the growth in the animals during the exposure period, and also to compensate for animal-to-animal variability in lung size, the parameters that were expressed in units of air flow or volume were normalized for each animal by its forced expiratory volume. The units for air flow data are thus transformed into multiples of the forced vital capacity per second and for the volume data the units are fractions of the forced vital capacity. In order to carry out a similar normalization of the forced vital capacity, this value for each animal was divided by that same animal's total lung capacity and accordingly, the units become a fraction of the total lung capacity.

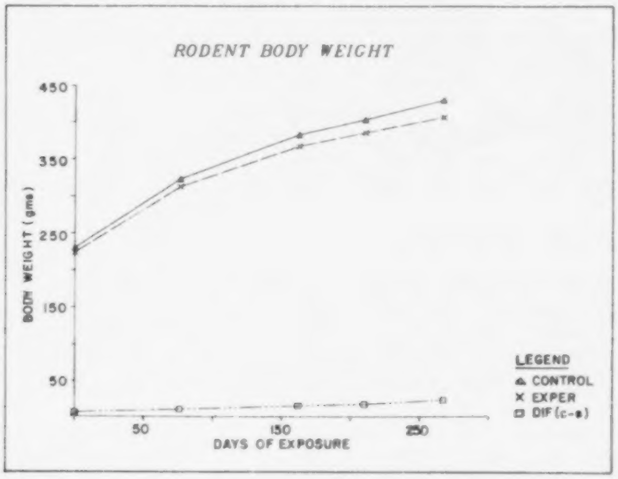

Figure 5A Body weight of the diesel exposed (X) and control (▲) animals vs. days on exposure regimen. The bottom curve (□) is the difference between the means of the two groups (control - experimental).

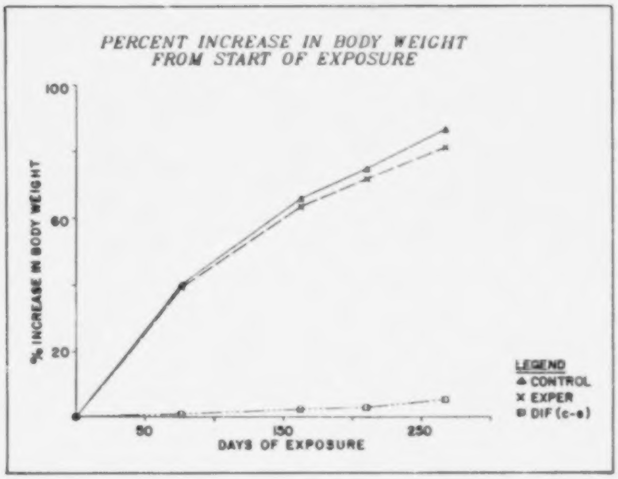

Figure 5B Percent increase in body weight for diesel exposed (X) and control (Δ) rats vs. the amount of time on exposure regimen. The bottom curve (□) is the difference between the mean percentage increases of the two groups (control - experimental).

Statistical Evaluation Tests for statistically significant differences between the control and experimental groups were carried out using analysis of variance. P values of 0.01 or less were accepted as indicating statistical significance.

Animals and Exposure Regimen

Fifty juvenile male Fischer-344 rats were randomly divided into two groups. The experimental group was placed in a 10 m^3 exposure chamber and exposed to diesel exhaust of a General Motors 5.7 liter engine produced by Oldsmobile diluted with clean air at a ratio of 1:40. This resulted in a diesel particulate concentration of 1500 $\mu g/m^3$. These animals were exposed to the exhaust for 20 hours per day, 5-1/2 days per week. Description of the inhalation chamber, diesel engine operating conditions and characterization of the inhalation chamber atmospheres have been described in detail elsewhere [10,11]. The control animals were placed in a similar chamber but exposed to clean, filtered air. Both groups of animals were given food and water *ad libitum*.

RESULTS

The data presented are the results of testing the animals after approximately 11, 23, 30 and 38 weeks on the exposure regimen. Table 1 displays the data without being normalized. Table 2 presents the normalized data with the results of statistical evaluation. There was no significant difference between control and experimental values for any of the parameters. Figures 6-9 display graphically some of the more commonly examined pulmonary function parameters presented in Table 2 as a function of the number of days on the diesel exhaust exposure regimen. The bottom curve in each graph represents the difference between the means at each data point (control value-experimental). These graphical representations help to more clearly see the lack of discrepancy between the control and experimental data. Figure 10 shows the lung volumes of inspiratory reserve, tidal volume and expiratory reserve as percentages of the forced vital capacity. Clearly, the differences between the control and experimental data are never more than a few percent. Figure 11 shows the total lung capacity divided by percentage into its constituents of inspiratory capacity and functional residual capacity. Again, little difference can be seen between the two groups of animals.

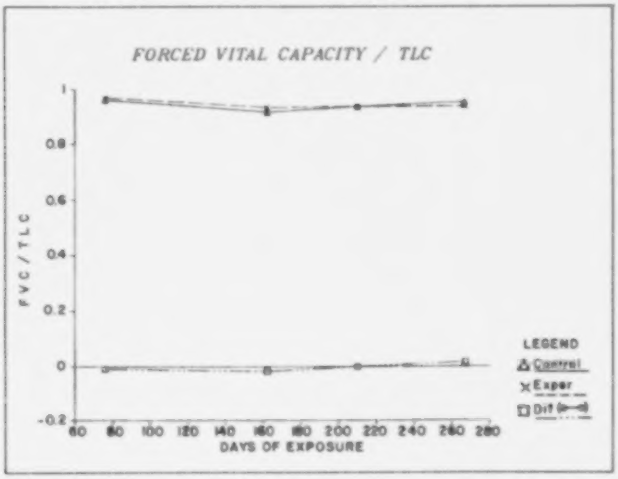

Figure 6 Forced vital capacity of diesel exposed (X) and control (△) animals as a fraction of each animal's total lung capacity vs. the amount of time on the diesel exposure regimen. The bottom curve (□) is the difference between the means of the two groups (control-experimental).

Table 1

EFFECT OF CHRONIC DIESEL EXHAUST INHALATION[1] ON PULMONARY FUNCTION PARAMETERS IN FISCHER-344 RATS

		Test Run 1[2]		Test Run 2[3]		Test Run 3[4]		Test Run 4[5]	
		Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental
SPONTANEOUS BREATHING	Weight (g)	322.5 ± 3.7[6]	312.1 ± 4.6	381.5 ± 3.3	366.1 ± 4.8	401.9 ± 4.1	384.5 ± 5.0	429.2 ± 4.5	405.6 ± 5.2
	Tidal volume (mL)	1.9 ± 0.04	1.8 ± 0.04	1.7 ± 0.04	1.6 ± 0.04	1.6 ± 0.03	1.5 ± 0.02	1.6 ± 0.03	1.5 ± 0.03
	Peak-to-peak transpulmonary pressure (cmH$_2$O)	5.9 ± 0.2	5.9 ± 0.2	5.4 ± 0.2	5.5 ± 0.1	7.0 ± 0.2	6.7 ± 0.2	7.0 ± 0.2	6.4 ± 0.2
	Dynamic compliance (mL/cmH$_2$O)	0.34 ± 0.01	0.32 ± 0.01	0.32 ± 0.01	0.30 ± 0.01	0.23 ± 0.01	0.34 ± 0.11	0.23 ± 0.01	0.23 ± 0.01
	Peak inspiratory flow (mL/sec)	7.8 ± 0.2	7.8 ± 0.2	6.4 ± 0.2	6.2 ± 0.2	6.5 ± 0.2	6.0 ± 0.1	6.3 ± 0.1	5.9 ± 0.1
	Peak expiratory flow (mL/sec)	15.1 ± 0.5	14.8 ± 0.5	14.5 ± 0.3	14.1 ± 0.3	14.7 ± 0.3	13.6 ± 0.20	14.0 ± 0.3	13.3 ± 0.3
	Functional residual capacity (mL)	1.57 ± 0.09	1.57 ± 0.07	1.62 ± 0.08	1.45 ± 0.06	1.49 ± 0.06	1.49 ± 0.06	1.61 ± 0.07	1.64 ± 0.05
FORCED MANEUVER	Forced expiratory volume - .1 sec (mL)	8.05 ± 0.22	7.79 ± 0.14	8.54 ± 0.16	8.15 ± 0.14	8.20 ± 0.11	7.59 ± 0.13	8.55 ± 0.16	8.25 ± 0.14
	Forced vital capacity (mL)	9.83 ± 0.36	9.80 ± 0.21	11.05 ± 0.25	10.42 ± 0.22	10.00 ± 0.17	9.14 ± 0.20	10.43 ± 0.27	10.06 ± 0.26
	Inspiratory capacity (mL)	8.70 ± 0.34	8.69 ± 0.19	10.4 ± 0.25	9.67 ± 0.23	9.27 ± 0.18	8.32 ± 0.21	9.41 ± 0.28	9.22 ± 0.28
	Expiratory reserve (mL)	1.20 ± 0.06	1.11 ± 0.07	0.61 ± 0.04	0.75 ± 0.06	0.73 ± 0.04	0.83 ± 0.04	1.02 ± 0.07	0.88 ± 0.06
	Peak expiratory flow rate (mL/sec)	109.9 ± 2.2	103.9 ± 1.8	115.1 ± 1.9	109.2 ± 2.1	115.9 ± 2.0	110.4 ± 1.8	127.3 ± 1.7	116.7 ± 1.8
	% Vital capacity at PEFR	62.4 ± 0.8	62.0 ± 1.7	66.3 ± 0.7	67.2 ± 0.7	63.6 ± 0.7	63.4 ± 0.7	62.0 ± 0.8	63.6 ± 0.8
	Maximum expiratory flow at 50% vital capacity (mL/sec)	103.7 ± 2.2	94.9 ± 2.7	105.6 ± 1.8	100.3 ± 2.1	108.8 ± 2.0	101.9 ± 1.7	120.8 ± 1.9	109.8 ± 1.6
	Maximum expiratory flow at 40% vital capacity (mL/sec)	92.8 ± 2.4	84.7 ± 3.3	92.2 ± 2.0	91.2 ± 2.3	102.6 ± 1.7	94.3 ± 2.0	112.6 ± 1.7	103.3 ± 1.1
	Maximum expiratory flow at 20% vital capacity (mL/sec)	54.8 ± 2.2	51.5 ± 2.1	50.3 ± 1.5	52.3 ± 1.7	57.3 ± 1.9	56.0 ± 2.6	58.7 ± 1.8	60.4 ± 1.8
	Mean expiratory flow 50-0% vital capacity (mL/sec)	51.9 ± 1.1	47.2 ± 1.3	52.6 ± 0.9	50.3 ± 1.1	54.5 ± 1.1	51.2 ± 0.9	61.1 ± 1.0	54.8 ± 0.9
	(MEF$_{25}$)-(MEF$_{50-0}$)(mL/sec)	12.7 ± 1.8	12.9 ± 1.8	8.5 ± 1.3	13.0 ± 1.6	16.9 ± 1.5	18.2 ± 2.8	14.1 ± 1.8	20.3 ± 2.0
	Inspiratory reserve volume (mL)	6.80 ± 0.32	6.87 ± 0.19	8.77 ± 0.23	8.06 ± 0.23	7.67 ± 0.18	6.86 ± 0.20	7.9 ± 0.27	7.7 ± 0.28
	Total lung capacity (mL)	10.28 ± 0.39	10.16 ± 0.23	12.08 ± 0.26	11.26 ± 0.27	10.73 ± 0.19	9.81 ± 0.21	11.02 ± 0.31	10.80 ± 0.28
	Residual volume (mL)	0.71 ± 0.12	0.60 ± 0.07	1.04 ± 0.08	0.97 ± 0.09	0.77 ± 0.06	0.70 ± 0.06	0.70 ± 0.08	0.79 ± 0.06

[1] Diesel exhaust particulate matter concentration = 1,500 g/m^3. Animals exposed 20 hrs/day, 5-1/2 days/week.
[2] Mean number of days on exposure regimen = 76.
[3] Mean number of days on exposure regimen = 162.
[4] Mean number of days on exposure regimen = 210.
[5] Mean number of days on exposure regimen = 267.
[6] Standard error of the mean.

n (Control) = 25
n (Experimental) = 25

Table 2

EFFECT OF CHRONIC DIESEL EXHAUST INHALATION[1] ON PULMONARY FUNCTION PARAMETERS IN FISCHER-344 RATS

DATA EXPRESSED IN UNITS OF FLOW OR VOLUME HAVE BEEN NORMALIZED

Parameter	Test Run 1[2]		Test Run 2[3]		Test Run 3[4]		Test Run 4[5]		
	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental	p[6]
DATA NOT NORMALIZED									
Peak-to-peak transpulmonary pressure (cmH_2O)	5.9 ± 0.2	5.9 ± 0.2[7]	5.4 ± 0.2	5.5 ± 0.1	7.0 ± 0.2	6.7 ± 0.2	7.0 ± 0.2	6.4 ± 0.2	N.S.
Dynamic compliance (mL/cmH_2O)	0.34 ± 0.01	0.32 ± 0.01	0.32 ± 0.01	0.30 ± 0.01	0.23 ± 0.01	0.34 ± 0.11	0.23 ± 0.01	0.23 ± 0.01	N.S.
% Vital capacity at PEFR	62.4 ± 0.8	62.0 ± 1.7	66.3 ± 0.7	67.2 ± 0.7	63.6 ± 0.7	63.4 ± 0.7	62.0 ± 0.8	63.6 ± 0.8	N.S.
$(MEF_{25})-(MEF_{50-0})$(mL/sec)	12.7 ± 1.8	12.9 ± 1.8	8.5 ± 1.3	13.0 ± 1.6	16.9 ± 1.5	18.2 ± 2.8	14.1 ± 1.8	20.3 ± 2.0	N.S.
DATA NORMALIZED BY FORCED VITAL CAPACITY									
Tidal volume	0.20 ± 0.006	0.19 ± 0.005	0.15 ± 0.003	0.16 ± 0.004	0.16 ± 0.003	0.16 ± 0.003	0.15 ± 0.003	0.15 ± 0.005	N.S.
Peak inspiratory flow	0.81 ± 0.03	0.80 ± 0.02	0.58 ± 0.01	0.60 ± 0.02	0.66 ± 0.02	0.66 ± 0.02	0.61 ± 0.02	0.60 ± 0.02	N.S.
Peak expiratory flow	1.57 ± 0.06	1.52 ± 0.05	1.33 ± 0.03	1.36 ± 0.03	1.48 ± 0.04	1.50 ± 0.04	1.36 ± 0.03	1.34 ± 0.05	N.S.
Functional residual capacity	0.16 ± 0.009	0.16 ± 0.008	0.15 ± 0.008	0.16 ± 0.007	0.15 ± 0.006	0.17 ± 0.007	0.15 ± 0.006	0.16 ± 0.006	N.S.
FEV/FVC x 100	82.0 ± 1.0	79.8 ± 1.2	77.6 ± 0.7	78.7 ± 0.9	82.2 ± 0.6	83.3 ± 0.8	82.3 ± 0.7	82.5 ± 0.8	N.S.
Inspiratory capacity	0.88 ± 0.011	0.89 ± 0.006	0.94 ± 0.004	0.93 ± 0.005	0.93 ± 0.004	0.91 ± 0.005	0.90 ± 0.007	0.91 ± 0.007	N.S.
Expiratory reserve	0.12 ± 0.006	0.11 ± 0.006	0.06 ± 0.004	0.07 ± 0.006	0.07 ± 0.004	0.09 ± 0.005	0.10 ± 0.007	0.09 ± 0.006	N.S.
Peak expiratory flow rate	11.33 ± 0.37	10.66 ± 0.20	10.49 ± 0.20	10.56 ± 0.24	11.64 ± 0.23	12.15 ± 0.23	12.34 ± 0.27	11.74 ± 0.28	N.S.
Maximum expiratory flow at 50% V.C.	10.70 ± 0.36	9.75 ± 0.31	9.63 ± 0.20	9.70 ± 0.24	10.93 ± 0.23	11.24 ± 0.26	11.71 ± 0.29	11.05 ± 0.27	N.S.
Maximum expiratory flow at 40% V.C.	9.56 ± 0.33	8.72 ± 0.37	8.41 ± 0.21	8.83 ± 0.26	10.29 ± 0.18	10.41 ± 0.28	10.90 ± 0.21	10.41 ± 0.25	N.S.
Maximum expiratory flow at 20% V.C.	5.62 ± 0.24	5.32 ± 0.26	4.59 ± 0.14	5.06 ± 0.18	5.76 ± 0.21	6.19 ± 0.30	5.66 ± 0.16	6.10 ± 0.24	N.S.
Mean expiratory flow 50-0% V.C.	5.35 ± 0.18	4.85 ± 0.15	4.80 ± 0.10	4.86 ± 0.13	5.47 ± 0.12	5.64 ± 0.13	5.92 ± 0.13	5.51 ± 0.14	N.S.
Inspiratory reserve volume	0.69 ± 0.013	0.70 ± 0.008	0.80 ± 0.005	0.77 ± 0.008	0.77 ± 0.006	0.75 ± 0.007	0.75 ± 0.009	0.77 ± 0.010	N.S.
Total lung capacity	1.05 ± 0.016	1.04 ± 0.015	1.10 ± 0.008	1.08 ± 0.013	1.07 ± 0.005	1.07 ± 0.007	1.06 ± 0.009	1.07 ± 0.006	N.S.
Residual volume	0.07 ± 0.013	0.06 ± 0.008	0.10 ± 0.008	0.09 ± 0.009	0.08 ± 0.005	0.08 ± 0.007	0.07 ± 0.007	0.08 ± 0.006	N.S.
DATA NORMALIZED BY TOTAL LUNG CAPACITY									
Forced expiratory volume	0.960 ± 0.013	0.968 ± 0.017	0.911 ± 0.007	0.928 ± 0.013	0.932 ± 0.005	0.932 ± 0.006	0.949 ± 0.008	0.933 ± 0.005	N.S.

[1] Diesel exhaust particulate matter concentration = 1,500 g/m^3. Animals exposed 20 hrs/day, 5-1/2 days/week.

N.S. = Not statistically significant $p > .01$

n (Control) = 25

n (Experimental) = 25

[2] Mean number of days on exposure regimen = 76.

[3] Mean number of days on exposure regimen = 162.

[4] Mean number of days on exposure regimen = 210.

[5] Mean number of days on exposure regimen = 267.

[6] Analysis of variance performed comparing control to experimental.

[7] Standard error of the mean.

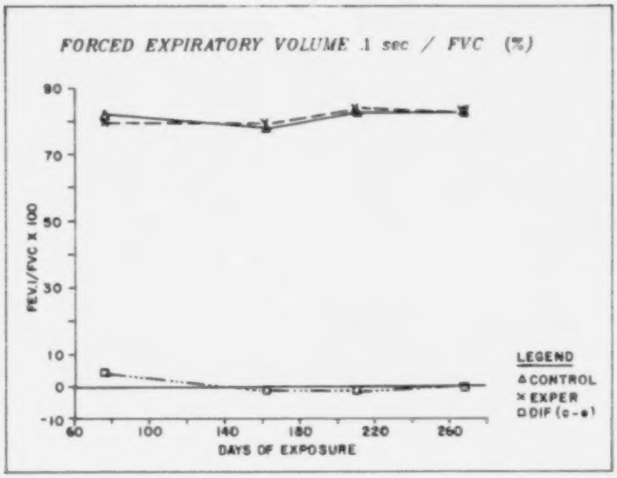

Figure 7 Forced expiratory volume (.1 seconds) of diesel exposed (X) and control (Δ) animals as a percent of each animal's forced vital capacity. Bottom curve (□) is the difference between the means of the two groups (control - experimental).

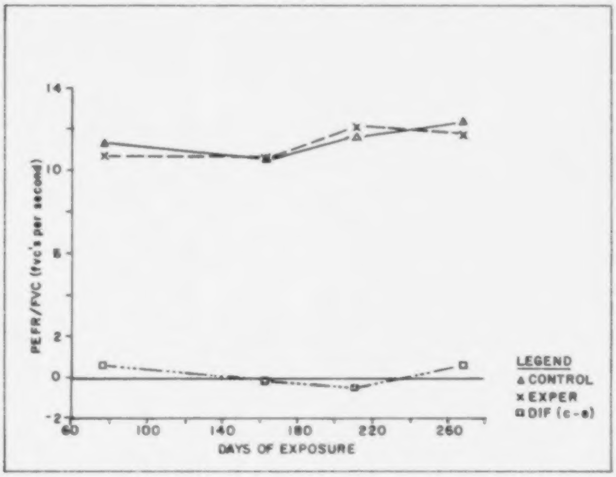

Figure 8 Peak expiratory flow rate of diesel exposed (X) and control (Δ) animals normalized by dividing the value obtained from each animal by that same animal's forced vital capacity. The bottom curve (□) is the difference between the means of the two groups (control - experimental).

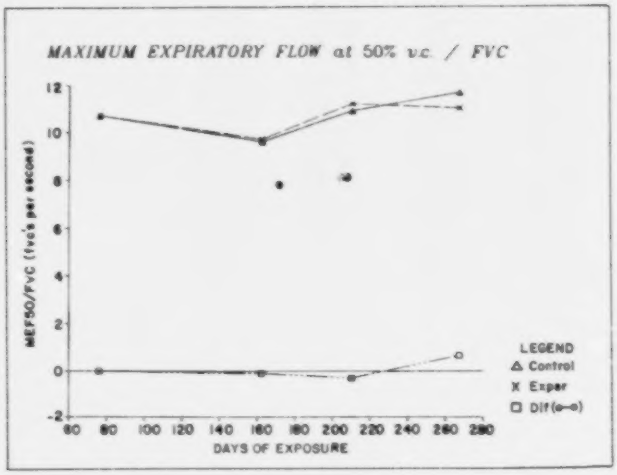

Figure 9 Maximum expiratory flow rate at 50% of vital capacity for diesel exposed (X) and control (△) rats normalized by dividing each animal's data by its forced vital capacity. Bottom curve (□) is the difference between the means of the two groups (control-experimental).

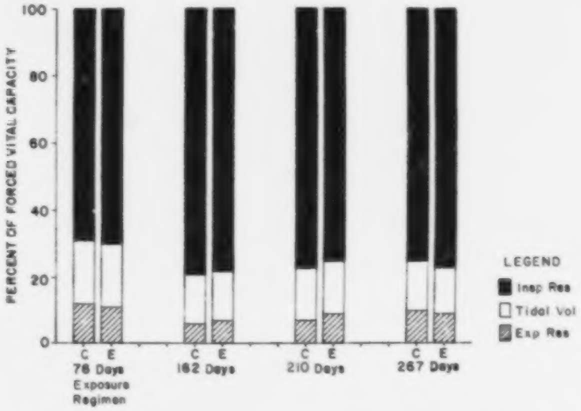

Figure 10 Inspiratory reserve (INS RES), tidal volume (TIDAL VOL) and expiratory reserve (EXP RES) of control (C) and diesel exposed experimental (E) rats expressed as a percentage of forced vital capacity (FVC).

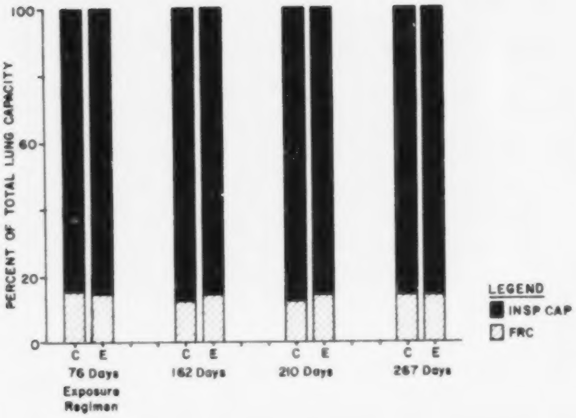

Figure 11 Inspiratory capacity (INSP CAP) and functional residual capacity (FRC) of control (C) and diesel exposed experimental (E) rats expressed as a percentage of total lung capacity (TLC).

DISCUSSION AND CONCLUSIONS

This technique for noninvasive pulmonary function testing of rodents appers to be well suited for ascertaining the potential effect that chronic inhalation of pollutants may have on the lung's mechanics and volumes. It offers the experimental advantage of following the same animal's responses many times over the entire exposure period. Thus, the variability introduced by testing a different group of animals at each point of time is eliminated and trends in the data may be more clearly seen. In spite of this advantage, we cannot detect any serious alteration of the lung's performance induced by the chronic inhalation of the diesel exhaust. In addition, the burden of the deposited diesel particles in the rat's lungs are far in excess of the deposits expected on the general population exposed to diesel exhaust on United States roads in the years to come [12]. These tests are limited in scope in that they do not serve as all encompassing indicators of the many possible alteration that could occur in the lung in response to diesel exhaust inhalation. They are similar to the more commonly used tests in clinical examination and evaluation of lung disease. Lastly, we should emphasize that we are looking for symptoms of chronic lung disease and this could take a longer time to develop and manifest itself in these tests. Therefore, the diesel exposure is continuing, the animals will be further tested and the additional data will be reported in the future.

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General Discussion

L. BUG: The technique is very impressive. Do you have any positive controls?

K. GROSS: Because of trying to get this thing geared up and going very quickly, we have not done the positive controls yet. We plan to do that shortly after the first of the year, but the best thing to do would be to refer you to the same technique done by Dr. Morely in which he does have positive controls and shows the system to be very functional.

MIHALKO: I was curious as to the inspiratory flow rate with the positive pressure reservoir. What was the inspiratory flow rate when you raised the lung volume to TLC?

K. GROSS: We have done this two different ways: one by using a metering valve so that it is very slow - about three or four milliliters per second. We have also done it without the metering valve. We haven't really measured what the inspiratory flow rate is if we do it without a metering valve. We didn't look at that for any data.

MIHALKO: What is the size of the tracheal tube you used?

K. GROSS: I don't have an actual numerical diameter. I can tell you that we make them ourselves. These are teflon and very thinned walled. We do everything we can to minimize obstruction or resistance by the tracheal tube, but I don't have the actual diameters for you.

MIHALKO: Have you measured the flow rate at minus 40 meters of water pressure through the tracheal tube alone?

K. GROSS: Yes, it is somewhat higher than anything we measured in the animal - the flow rate itself. We can put that on and go up at least another 10 percent than we do with the animals.

MIHALKO: Could you hypothesize why your peak expiratory flow rate occurs so late or at such a low lung volume?

K. GROSS: Perhaps the best way to describe that is to give you the actual number for some of the data. It occurs at about 42 percent of the vital capacity remaining. I am not sure how abnormal or normal it is compared to humans or other animals, but we have to remember that we have a system with a built-in resistance in the tubes and whatever we are looking at is basically the resistance of the system with the animal's own characteristics superimposed on top of that resistance.

G. EDWARDS: As a nonrespiratory physiologist, I have seen a lot of numbers in the literature as to what a rat or a mouse breaths. I noticed somebody here has 200 milliliters as the minute volume. I have seen from 90 to 300 milliliters. Could somebody give me an answer so I could make calculations in the future as to what that is?

K. GROSS: There are a lot of problems in measuring that type of thing in animals. The methods you have for measuring the respiratory rate and the manner with which they are inhaled usually involves restraints so the animals are highly excitable. Their adrenalin is pouring, and measurements are taken of the animals in very excited states. One way of approaching this is the way that Dr. Joe Mauderly has done. He has taken animals and trained them to put tneir faces against the breathing mask and they are rewarded for doing this, and they do it in an unexcited state. He has some data on that, but I don't recall offhand what he has. You have to remember that with our experiments, the animals are anesthesized. I don't have good data for that, and there is a real problem with taking that data.

B. STUART: You mentioned a high degree of variability among the individual animals. I didn't see any of these described on your graphs. Can you give us some idea of that, and in view of that fact, what sorts of tests will you use to determine wherher or not you are developing obstructive pulmonary disease?

K. GROSS: We don't. We find that when we normalize by the capacity that the data tightens up very nicely, and it seems to take into account the animal-to-animal variability. I think the important thing we are looking for is in the literature ranging, as you say, from below 100 mills to several hundred mills is, in fact, that the breathing patterns of these animals vary that much and it varies in humans that much too. There is no such thing as a normal breathing pattern. The breathing pattern varies tremendously. The number that you want is the breathing pattern under the condition that you are concerned about and most of us, in making deposition calculations in particular, are concerned about the breathing pattern inside the chamber at the time of exposure. Those measurements simply are not available at this time. The minute volume of an approximately 200 gram Fisher rat, unanesthesized, breathing through a normal breathing valve, is on the order of 200 mills a minute. But if that animal is sitting in a cage, semi-asleep as the cage sits on a laboratory cart, or something like that, it can easily be cut in half, or if someone walks into the room, it can easily double. This is a real problem and what we really don't have - and what we need are good values, averaging values over a period of time under actual exposure conditions.

that given a certain foresighted capacity the relationship of the other volumes and the other flows to each had changed. Usually that is what you look for. In chronic lung disease you see that the partitioning of the lung - volumes have changed somewhat, and we will continue to use these. These are the most sensitive things that I know of in small animals. We will probably add carbon monoxide defusing capacity to our battery of tests in the near future which will be another important indication for us.

B. STUART: Do you have any idea of the range of standard deviation among the animals?

K. GROSS: For most of these as it happened to work out, the standard error was approximately three to four percent of the mean, and so with an N of 25, about five times that would be the standard deviation, about 15 percent.

J. MAUDERLY: You are using the same catheter that we are; the internal diameter is 1.7 millimeters. We are all faced with the problem of breathing volume in animals. We are faced with it daily and what number do you use for breathing volumes. The reason that you see values reported

LYMPHATIC TRANSPORT OF INHALED DIESEL PARTICLES IN THE LUNGS OF RATS AND GUINEA PIGS EXPOSED TO DILUTED DIESEL EXHAUST

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ABSTRACT

Factors influencing the disposition of the inhaled diesel particles were studied by analyzing the deposition of radioactively labelled diesel particles in the respiratory system, by determining the specific function of alveolar cellular mechanisms in the primary defense against inhaled particles and by identifying the important role of the lymphatic system in the lung clearance of experimental animals exposed to diluted emissions from a diesel engine. Radioactive ^{131}Barium was used as a tracer of diesel particles and the deposition efficiency was determined to be 15 ± 6% of the inhaled dose in the Fischer 344 rat strain. The number of cells obtained by bronchial lavage increased significantly after a prolonged exposure to a concentration of 1500 $\mu g/m^3$ of diesel particles. The increased cell number was more than twofold, contained two distinct cell populations (alveolar macrophages and neutrophils) and represented a reactive mobilization of the defense mechanisms in the organism.

Light microscopy studies investigated the role of lymphatic transport of the particulate matter and revealed that the peribronchial and perivascular aggregates of lymphoid tissue contained diesel particles even after short exposure periods at low dose levels. With the increasing burden of particles in the respiratory system, the coloration of hilar and mediastinal lymph nodes continuously changed to gray and finally to dark black, depending upon the dose level and exposure. However, at all exposure levels, most of the diesel particles in the alveoli were phagocytized by an increased alveolar cellular defense and particle-containing macrophages were actively moving towards the mucociliary escalator or towards lymphatic channels leading to peribronchial lymphoid aggregates and bronchial or mediastinal lymph nodes.

In the lymph nodes, alveolar macrophages containing diesel particles were found mostly in the afferent subcapsular lymphatic vessels and marginal sinuses. In the later stages, cellular structure disintegrated and large aggregates of particulate matter were dispersed throughout the medullary cords with increasing accumulation towards the hilus. It is concluded that the lymphoid aggregates and lymphatic nodes play an important role in sequestering diesel particles or particle-containing phagocytizing cells and provide a pathway, in addition to the mucociliary clearance for particulate removal from the deep pulmonary region.

INTRODUCTION

It is widely accepted that the inhaled particles deposited in the respiratory system are removed from the alveolar and bronchial regions by a complex of mechanisms, generally called lung clearance. Continuous movement of the particles deposited on a thick carpet of bronchial mucus and transport by the rapid metachronal action of cilia on bronchial cells towards the oropharyngeal cavity, along with the scavenging phagocytary function of a special type of pulmonary cells called alveolar macrophages, have been considered the main factors in this process of "mucociliary escalator" clearance [1-8]. In addition, an effective role of the lymphatic system in the lung clearance process was postulated by several authors in the second half of the 19th century, but its importance has not yet been satisfactorily documented.

Diesel particulate emissions contain submicron size aerosols which can penetrate deeply into the respiratory system. In particular, the insoluble carbon core of the diesel particles provides easy identification of diesel particles in the pulmonary regions of the respiratory tract. Due to their specific appearance, diesel particles are easily recognizable, and their transport route can be easily followed by macroscopic and microscopic methods.

The investigations described in this publication concentrated, therefore:

1. on determining the deposition efficiency of radioactively labelled diesel particles in the lungs of Fischer 344 rats,
2. on the specific function of the pulmonary alveolar macrophages as the major protection mechanism in the primary defense against inhaled particles, and
3. on the important role of the lymphatic system in the mechanisms of lung clearance of diesel particles.

DEPOSITION OF RADIOACTIVELY LABELLED DIESEL PARTICULATES IN THE RESPIRATORY SYSTEM

The initial deposition and subsequent clearance of the particles in the lungs have a significant role in predetermining the site of their potential interactions with the cells of the respiratory system and need to be considered in any assessment of the potential health hazard of inhaled diesel emissions. Unfortunately, no information is available regarding the deposition efficiency of inhaled diesel particles. A theoretical deposition model proposed by the International Committee for Radiation Protection (ICRP) (Figure 1), has been constructed and used to predict the total and regional deposition of inhaled particles in man [9]. Based on the size of freshly-generated diesel particles, the model predicts that a total deposition of approximately 35% of diesel particles with 0.2 μm mass median diameter may be expected to occur, mostly in the deep (alveolar) pulmonary region. However, the validity of this predictive model for diesel particle deposition in animals has never been tested. Deposition efficiency of inhaled diesel particles was, therefore, determined experimentally in this study by exposing test animals to radioactively-tagged diesel particles. The "nose-only" inhalation exposure was followed by external gamma counting. The amount of radioactivity detected in the test animals after the exposure served as an indirect measure of the deposition of diesel particles.

Radioactive label containing fuel additive was synthesized for generation of radioactively tagged diesel particles. Since barium or calcium salts of sulfonic acid have been used as additives reducing smoke opacity of diesel exhaust, are soluble in diesel fuels at low concentrations and participate in the formation of diesel particulates, the gamma emitting isotope of ^{131}Ba (half-life of 11.7 days with photopeaks of 124, 216, 373, and 496 keV) was selected as the radioactive tracer and incorporated into barium dodecylbenzene sulfonate, Ba($C_{12}H_{25}$—⌬—SO_3)$_2$. The original compound was synthesized by the addition of barium

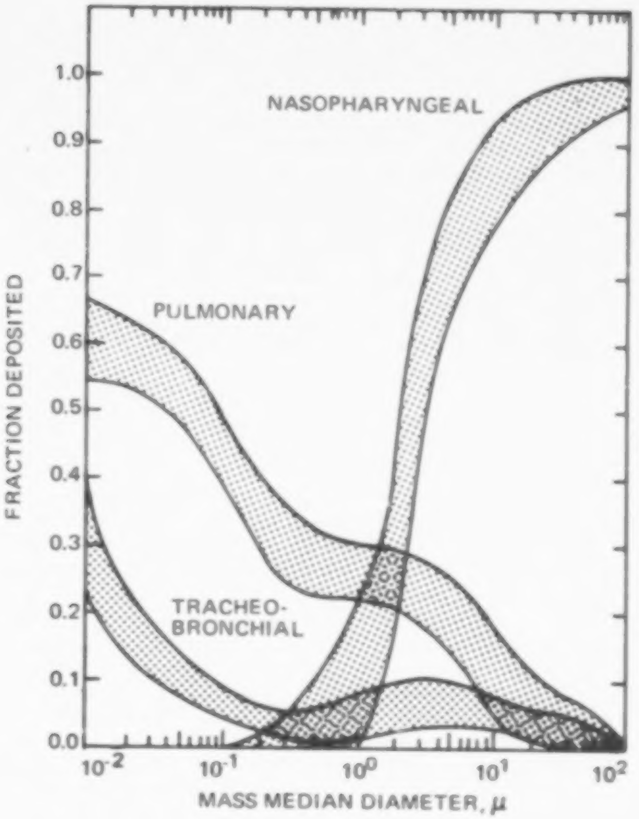

Figure 1 Fraction of particles deposited in the three respiratory tract compartments of man as a function of particle size, predicted by the ICRP Task Group on Lung Dynamics model [9].

hydroxide solution to dodecylbenzene sulfonic acid, purified, washed with isopropanol and dried at 1 mm Hg pressure. The radioactive ^{131}Barium dodecylbenzene sulfonate was then prepared by an isotope exchange reaction between ^{131}Barium chloride and barium dodecyl sulfonate in a binary mixture of 3:1 acetone-water solution. The final radioactive compound was added to the fuel at a barium concentration of 50 ppm and used in a 5.7 L GM diesel engine produced by Oldsmobile and operated under conditions comparable to those described for the long-term exposure experiments [11]. After thirty minutes exposure of the test animals to 10:1 diluted diesel exhaust, whole body counting was performed using NaI(Tl) scintillation detectors. Since the animals were exposed in a "nose-only" inhalation regime, the whole body count measurements are considered to reflect the total activity deposited via the inhalation route only. External contamination of the skin of the animal was measured and found to be negligible. Total deposition was calculated by dividing the initial activity in the rat as determined in the whole body counting detector by the estimated inhaled activity calculated as the product of the specific activity of the particles in the exposure chamber, the tidal volume, the respiratory frequency and the exposure duration.

The initial deposition efficiency of freshly generated diesel particles, determined by this method in 22 test animals, was found to be 15 ± 6% of the inhaled dose. Based on the deposition efficiency obtained experimentally on the Fischer 344 rats in this study, we can expect that the ICRP Task Group Model would have overestimated particle deposition by almost a factor of two for this species. Additional experiments are in progress to characterize deposition and clearance rates in more detail and to determine the appropriate correction factors for extrapolation to man [10].

PARTICULATE DEPOSITS IN THE RESPIRATORY SYSTEM AFTER LONG-TERM INHALATION EXPOSURES

In 1978, the Biomedical Science Department of the General Motors Research Laboratories initiated a chronic inhalation study to identify any potential adverse health effects to the respiratory system resulting from a long-term exposure to diluted diesel exhaust. Within this study, fifty-four male Fischer 344 rats (Charles River Breeding Laboratories) and fifty-four male Hartley albino guinea pigs (Charles River Breeding Laboratories) were exposed to 250, 750 and 1500 $\mu g/m^3$ of diesel exhaust for 5, 10, 15, 25, 35 and 45 weeks under the conditions described by Schreck *et al* [11]. Continuous exposures lasted 20 hrs/day, 5-1/2 days/week and the combination of the dose and time represent an enormously high burden of particulates applied to the experimental animals.

Immediately after each exposure period, a thoracotomy was performed in animals anesthetized by sodium pentobarbital (30-50 mg/kg I.P.) and the entire lung, carina and lymph nodes from the mediastinal regions were removed and fixed in neutral formalin for light microscopy. The sections were cut at 6 μm and were stained with hematoxylin and eosin, Verhoeff-Van Gieson stain, and Gomori's Silver Method for reticulin.

The tissue for transmission electron microscopy was fixed in Karnovsky's fixative at 4°C and dehydrated in alcohol and embedded in epon. The thick sections (0.5 μm) were cut on a Richert-OMU-3 microtome and stained with toluidine blue and examined under the light microscope. Fine sections (50 μm) were cut and stained with uranyl acetate and lead citrate and studied in a Zeiss EM10B electron microscope.

Table 1 shows a calculated comparative estimate of the maximum expected concentrations in the lungs of experimental animals after one year for different levels of exposure and indicates without any doubt that even for the lowest experimental concentration of 250 μg/m^3, the impact on the experimental animal has been at least 1000 times greater than that which can be expected for population groups continuously exposed on our roads in the year 2000. The exposure estimate for man is based on the best estimate of the regional annual mean concentration of 3.5 μg/m^3 for Chicago reported by Williams and Chock [12] earlier in this meeting.

Table 1

EXPECTED LUNG DOSES OF INHALED DIESEL PARTICLES

Diesel Aerosol Exposure (μg/m3)	Calculated Lung Retention Per Year Assuming 30% Dep. (μg)	Resulting Lung Concentration (mg/g)
RAT 250$^{(1)}$	5.000 μg	5
1.500$^{(1)}$	32.000 μg	32
6.000$^{(2)}$	60.000 μg	60
MAN 5$^{(3)}$	5.000	0.005

[1] 20 Hours/Day, 5 ½ Days/Week, Daily Ventilation = 0.3 M^3, Lung Wt. = 1.0 g.

[2] 8 Hours/Day, 7 Days/Week, Daily Ventilation = 0.3 M^3, Lung Wt. = 1.0 g.

[3] 24 Hours/Day, 7 Days/Week, Daily Ventilation = 10 M^3, Lung Wt. = 1 kg.

The administered dose has apparently exceeded the capacity of otherwise effective lung clearance mechanisms, and resulted in an excessive accumulation of particulate matter in the parenchyma, which was easily recognizable even under macroscopic examination. Figure 2 compares the inflated lungs of a Fischer 344 rat exposed for 35 weeks to a concentration of 1500 $\mu g/m^3$ with the control and clearly indicates a rapid accumulation of black material in the lungs. The excessive exposure resulted in a complete change of color of the lung from pink-white to dark, solid black. Surprisingly, traces of similar discoloration at a much lower level can be observed even in the lowest exposure group of 250 $\mu g/m^3$ where the first change in the color of the exposed lung occurred after 15 weeks of exposure (Figure 3). Once the normal clearance mechanisms were exceeded, the continuing exposure resulted in increasing intensity of the black coloration. Table 2 presents a semi-quantitative subjective evaluation of the changes in lung coloration with increasing intensity and length of exposure in both tested experimental species.

Table 2

MACROSCOPICALLY OBSERVED COLORATION OF LUNG
(Subjective Evaluation Immediately After Lung Inflation)

Time of Exposure in Weeks	Rat 250	Rat 750	Rat 1500 $\mu g/m^3$	Guinea Pig 250	Guinea Pig 750	Guinea Pig 1500 $\mu g/m^3$
5	0	±	+	±	+	++
10	±	+	++	±	++	+++
15	±	++	+++	+	++	+++
25	+		++++	++		++++
35	++		++++	++		++++
45	++		++++	++		++++

± Slight change in color
+ Grayish
++ Gray
+++ Black
++++ Solid Black

Microscopic pictures confirm the macroscopic observations, with the differences between high and low levels of exposure being even more startling. Lung sections from the control and exposed animals can be compared and indicate the presence of isolated cells in the exposed animals which are freely floating in the alveolar lumen and are filled with large numbers of diesel particulates (pulmonary alveolar macrophages).

In a macrophage obtained from an animal exposed to diesel emissions, low or high numbers of aggregated particles can be easily identified inside the cytoplasmic membrane or in lysosomes, depending on the level and duration of exposure. The migrating cell appears on scanning electron microscopy

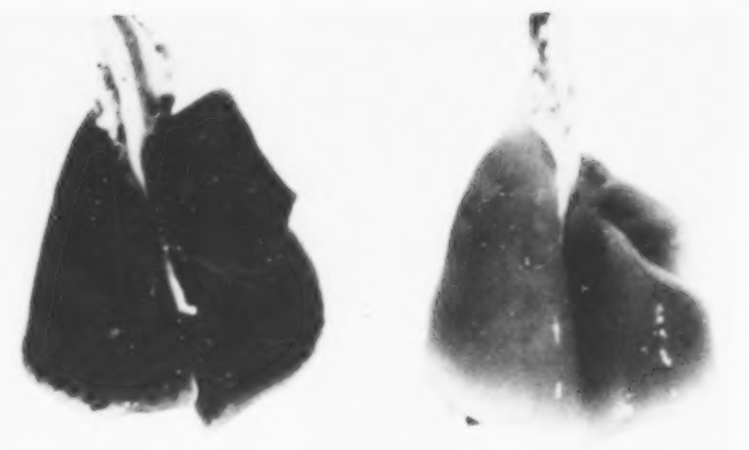

Figure 2 Macroscopic lung appearance from a rat exposed to 1500 μg/m³ for 35 weeks. On the right, a lung from a filtered air exposed animal.

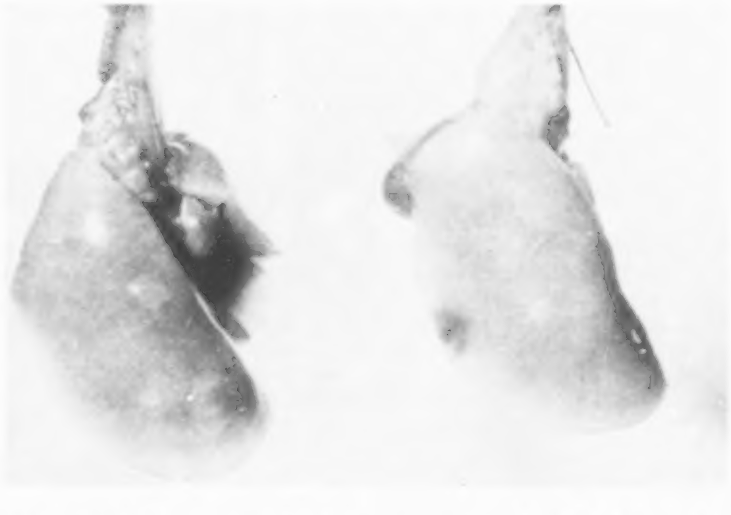

Figure 3 Macroscopic lung appearance from a rat exposed to 250 μg/m³ for 15 weeks (left). On the right, a lung from a rat exposed to filtered air.

as a solitary sphere with numerous microvilli on the surface. On transmission electron microscopy, this cell usually shows a large number of lysosomes and various-sized vacuoles. The Golgi apparatus appears active and the number and size of mitochondria remains unchanged (Figure 4).

It is important to note that in the early stages of low level exposures, free diesel particles in the lung parenchyma are rarely found, so effective is the scavenging function of the macrophages in the pulmonary alveoli (Figures 5,6).

Furthermore, a simple comparison of the early stages of low level exposures with the higher concentrations and longer exposure times indicates clearly -- even without a morphometric evaluation -- that the number of mobilized phagocytic cells increases proportionately with the increasing burden of inhaled particles (Figure 7).

FUNCTION OF THE ALVEOLAR CELLULAR DEFENSE MECHANISMS

The pulmonary alveolar macrophage is the principal resident mononuclear phagocyte of the lung and its function in the primary defense against inhaled particulate matter has been widely documented and recently reviewed [13].

It has been well established that this important mechanism of cellular defense is not of local origin. In contrast to original claims that the pulmonary alveolar macrophage (PAM) is a mobilized cell which emigrated from the alveolar interstitium, recent studies with radioactive thymidine seem to indicate that the alveolar macrophage is a transformed monocyte which originates from bone marrow, and, like the other tissue macrophages, is transported in the form of a monocyte to the peripheral organ where it becomes a fixed or free cell with phagocytic function.

Since pulmonary alveolar macrophages are freely moving in the alveoli, they can be easily washed out of the pulmonary airways and harvested from the respiratory system by broncho-pulmonary lavage, applied as a terminal or even as a non-invasive diagnostic technique. The technique was first described in 1961 by Myrvik *et al* [14] and since that time, the broncho-pulmonary lavage became widely used in many experimental, and even human, studies.

Particularly, the technique permitted demonstration that the number of macrophages in the alveolar lumen is highly reactive to the presence of dust particles in the respiratory system. Thus, Pratt *et al* [15] demonstrated that the

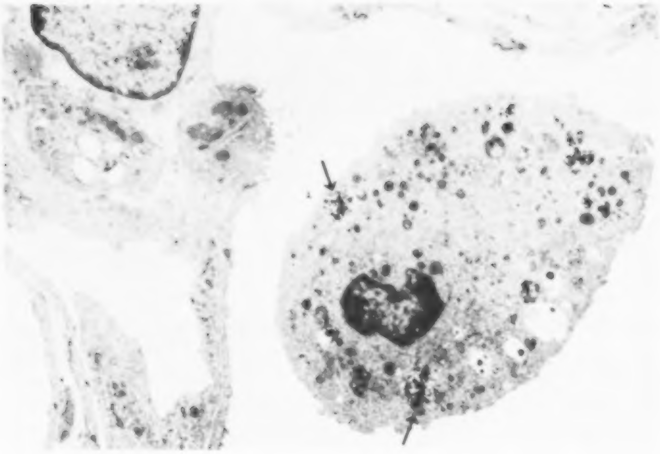

Figure 4 Electron micrograph of an alveolar macrophage in the alveolar lumen. Few vacuoles contain diesel particles (↑).

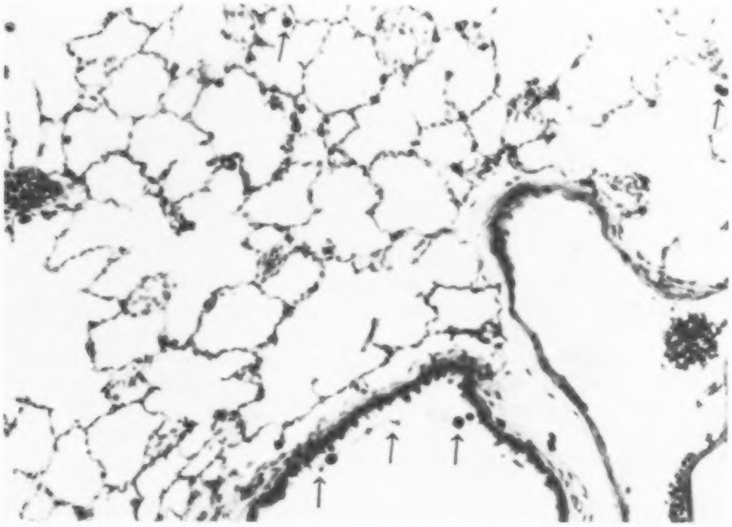

Figure 5 Micrograph from a diesel-exposed rat lung (250 μg/m³) for 10 weeks. Isolated pulmonary alveolar macrophages are present in the alveoli and in a bronchiole (↑).

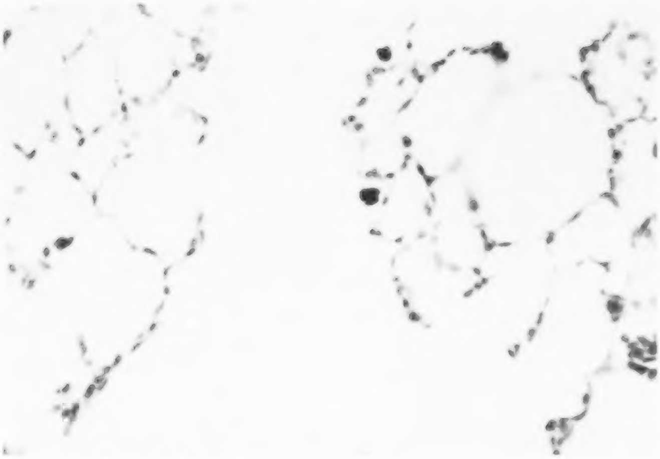

Figure 6 Isolated pulmonary alveolar macrophages are filled with dense diesel particulate aggregates. No free particulates can be identified in the alveolar interstitium.

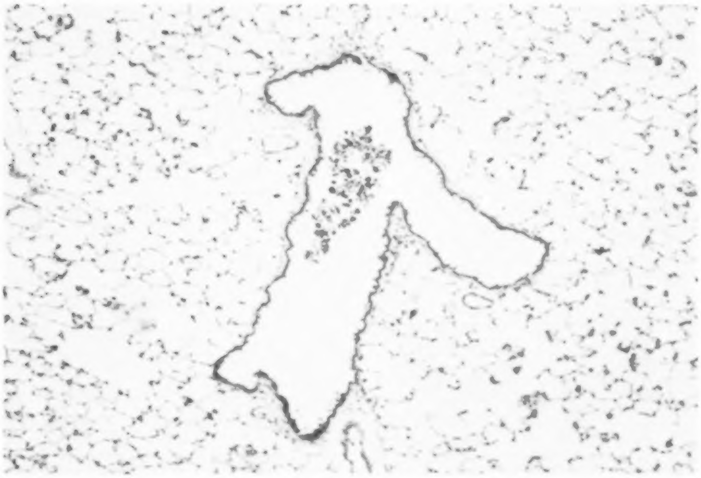

Figure 7 Micrograph from a diesel exposed rat lung (1500 $\mu g/m^3$) for 10 weeks. There are numerous macrophages in the alveoli. Proteinaceous debris, including a large number of macrophages with phagocytized diesel particles, is present in the lumen of a bronchiole.

number of the phagocytizing cells and their burden of particles is greater in smokers than in nonsmokers and recognized that the mobilization of macrophages represents an adaptive reaction to the environment. Recent studies by Matulionis [16,17] claim that inhalation of cigarette smoke rapidly induces increased DNA concentrations in lung. Since this reaction is specific for pulmonary tissue only, it is interpreted as a mobilization and recruitment of alveolar macrophages.

Obviously, such an adaptive reaction would be of cardinal importance for the clearance of diesel particles from the lung, and the effects of different levels of exposure on mobilization of pulmonary alveolar macrophages were therefore analyzed in the present study. After 6 to 11 months of exposure, groups of six male Fischer 344 rats were anesthetized and sacrificed by pneumothorax and lavaged with the lung *in situ*. The cells which were obtained were analyzed for kind, number, size, biochemistry, and morphology. Figure 8 shows the size distribution histogram of the cells from lung lavage of rats which have been exposed for 6 months to concentrations of 0, 250, or 1500 μg/m^3 of diesel particulates.

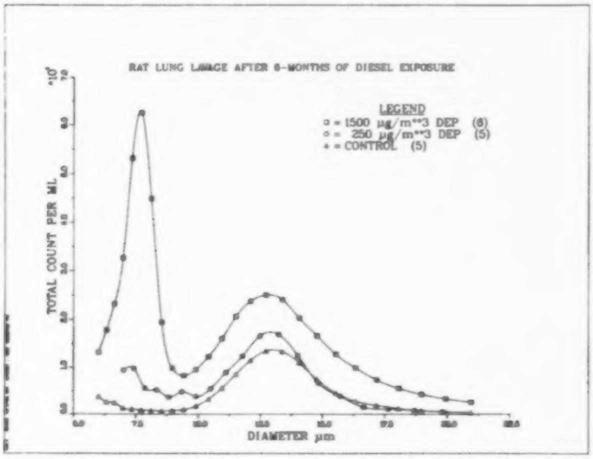

Figure 8 Cell size distribution in rat lung lavage after different levels of exposure to diesel particulates.

The modal size distribution of the harvested macrophages in the control group has a cell diameter of approximately 13 micrometers--a size typical for a functioning alveolar macrophage. Furthermore, the identity of this population as pulmonary alveolar macrophages was confirmed by a histological examination of the cells. At an exposure concentration of 1500 $\mu g/m^3$, the increase in macrophage population is more than twofold and denotes a significant mobilization of the defense mechanisms in the organism (Table 3). However, concurrent with this quantitative change, a qualitative change also occurs. The size analysis indicated clearly that another population of cells appears in the exposed group and that these new cells are only half of the size of the normal alveolar macrophage, i.e., about 8 micrometers in diameter. The mobilization of the new cell population is also sensitive to the dose of particles deposited in the lung. It may be barely discernable in the 250 $\mu g/m^3$ exposure group, but it represents a population comparable with the stimulated alveolar macrophages when the exposure level is increased to 1500 $\mu g/m^3$.

Table 3

COMPOSITION OF CELLS OBTAINED BY LUNG LAVAGE OF RATS EXPOSED TO DIESEL EXHAUST PARTICULATES
(Millions of Cells per Animal)

	Duration of Inhalation Exposure					
	6 months			11 months		
	Diesel Exhaust Particulate ($\mu g/m^3$)					
	0	250	1500	0	250	1500
Alveolar Macrophages	5.4 ± 0.5	5.7 ± 1.3	13.5 ± 2.5 S	7.2 ± 1.7	7.1 ± 1.1	15.4 ± 1.6 S
Neutrophils	0.2 ± 0.1	0.5 ± 0.3	7.5 ± 2.0 S	0.3 ± 0.2	0.7 ± 0.4	20.0 ± 4.3 S

S - Significance at $P < 0.01$

Cytological identification with the help of Papanicolaou and Wright Giemsa stains shows that the new population is distinctly different from the pulmonary alveolar macrophages (Figure 9). Instead of a large cell with a dense nucleus and basophilic cytoplasm, which is a picture typical for the macrophage, the new cell population has a pale, lobed nucleus and distinctly neutrophilic cytoplasm (Figure 10). In cell culture, the new cells are viable, mobile, and have full phagocytic function, all signs of the cytology typical for neutrophilic white blood cells. The

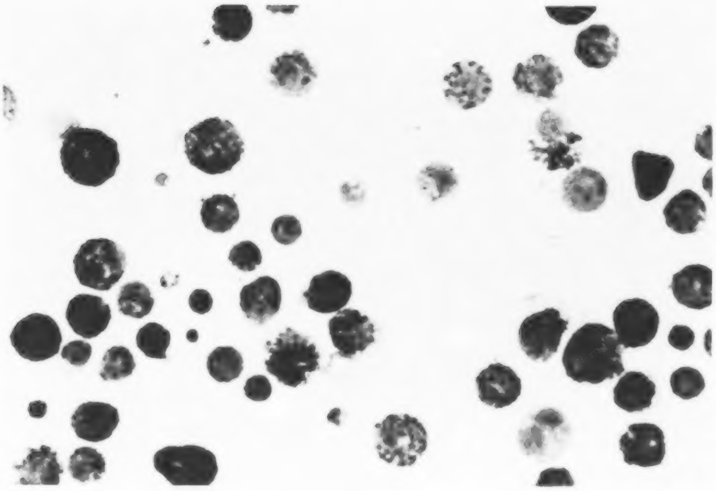

Figure 9 Population of lavaged macrophages from a rat exposed to diesel exhaust at 250 µg/m³ for 45 weeks. The macrophages contain numerous diesel particles.

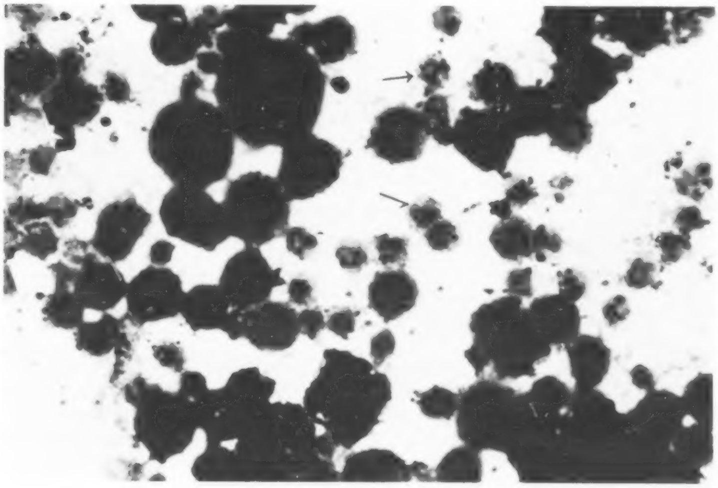

Figure 10 Cytological identification of two distinct populations of cells lavaged from a rat exposed to 1500 µg/m³ for 45 weeks. Smaller sized cells have a pale, lobed nucleus and distinctly neutrophilic cytoplasm (↑). Both types of cells contain diesel particles.

phagocytic function can be ascertained by the black particulate matter contained in cells from advanced stages of diesel exposed animals.

The neutrophils assist the monocyte-derived alveolar macrophages in their function of clearing the alveolar lumen of diesel particles and have been also observed after stimulation by dusts other than diesel particulates [18]. Therefore, it may be considered as another effective phase of the cellular defense in the organism. However, neutrophils are never present in large numbers in normal lung lavage and the stimulus responsible for the cellular activation of macrophages and neutrophils still remains unidentified. Although the physico-chemical properties of the particles have been claimed to be responsible for this phenomenon [18] and a specific stimulus of carbon-derived particles for neutrophil mobilization emphasized, the specific role of neutrophils in initiating fibro-proliferative or inflammatory processes certainly needs to be studied further.

ROLE OF THE LYMPHATIC SYSTEM IN THE LUNG CLEARANCE OF DIESEL PARTICULATES

In the preceding section, we have described the function of the alveolar macrophage as a pulmonary scavenger which can effectively clean the alveolar space of diesel particles. If the phagocytized particles are microbes, the macrophage has an effective lysosomal system which results in a complete degradation of the invading micro-organism. However, when the phagocytized materials are not of biological origin and consist of insoluble carbon particles, the fate of the deposited material is dependent on the efficacy of the lung clearance mechanisms.

The lining of the respiratory airways consists predominantly of a ciliated columnar epithelium and cells with hair-like vibrating cilia on their luminal sides which are present even in the smallest airways. The so-called "mucociliary escalator" starts, therefore, at the most distal point of the airways epithelium and provides an effective clearance mechanism for the phagocytes containing diesel particles (Figure 11). In this way, most of the macrophages laden with diesel particles are transported from the alveolar region and reach the oropharyngeal cavity to be disposed of via expectoration or passage through the gastro-intestinal tract before they can exert any biological action (Figure 12). However, not all of the alveolar macrophages use the escalator mechanism.

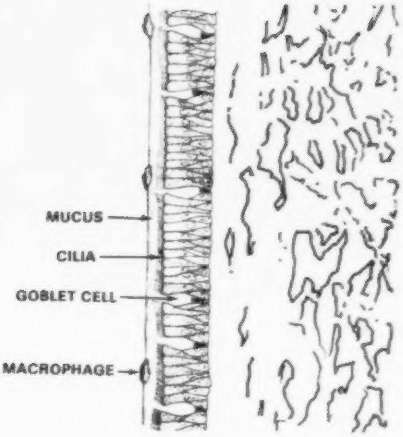

Figure 11 Schematic representation of the "mucociliary escalator" in the conducting airways showing macrophages on the surface of the mucous blanket.

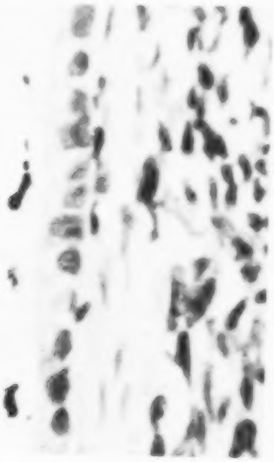

Figure 12 A section of a bronchus showing the transport of the diesel laden macrophages on the ciliated epithelium. Some macrophages on the ciliated epithelium. Some macrophages are free in the lumen.

Observations done on the diesel exposed animals indicate that in addition to this type of effective lung clearance, there may be another pathway through which the macrophage can leave the respiratory system. Shortly after the animals have been exposed to high concentrations of particles, macrophages can be found not only in the alveolar lumen where their number rapidly increases but very soon, free particulate material and a few macrophages appear also in the lumen of the lymphatic channels.

At the most peripheral part, the lymphatic system of the lung consists of diffuse lymphatic tissue in periarterial and peribronchial lymphatic sheets. Lymphatic fluid produced in this terminal part by ultrafiltration from blood is collected by lymphatic capillaries, transported in a centripetal direction through peribronchial lymphatic channels towards the pulmonary hilus, and filtered in hilar and other mediastinal lymph nodes before it returns into the general circulation via the thoracic duct. The lymph is essentially an ultrafiltrate of blood plasma and, in the resting state, contains only a very few cells, usually lymphocytes and/or free tissue macrophages. The situation, however, rapidly changes when the cellular tissue defense is activated.

Our studies indicate that particularly in the respiratory system of the exposed animals and after excessive exposures to diesel exhaust and mobilization of alveolar macrophages, cells laden with particulates are found near the peribronchial and periarterial lymphoid aggregates (Figure 13). With continuing exposure, diesel particle-containing macrophages or free particulate aggregates are identified even in large lymphatic vessels and in lymphatic nodes (Figure 14,15).

Lymph nodes function as an effective filter. The afferent lymphatic vessels bring lymph and macrophages with diesel particulates into the subcapsular or marginal sinuses, where the macrophages are moved through the outer and inner cortex into the medullary cord and medullary sinuses. It is usually the borderline between the medullary cord and medullary sinus which functions as a filter and where most of the organically coated particles are accumulated (probably by active phagocytosis by fixed monocytic macrophages) and retained in the node.

In this study, alveolar macrophages containing diesel particles were found mostly in the afferent subcapsular lymphatic vessels and marginal sinuses. In the later stages of exposure, cellular structure of macrophages with particles disintegrated and large aggregates of particulate matter were dispersed throughout the medullary cords with

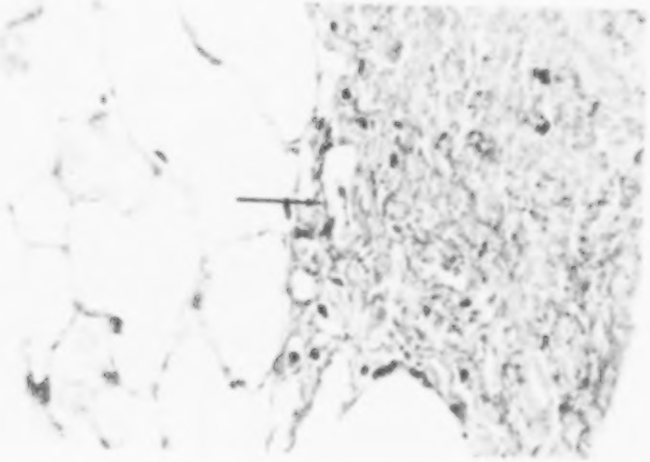

Figure 13 Accumulation of macrophages with diesel particles in a peribronchial lymphoid aggregate in a juxtabronchial region. Arrow indicates a lymphatic duct containing a diesel particle-laden macrophage.

Figure 14 Elongated macrophages stuffed with diesel particles are transported through a lymphatic vessel towards a peribronchial lymphoid aggregate.

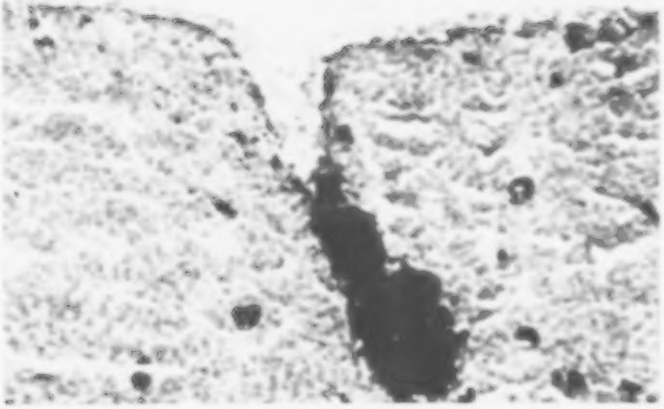

Figure 15 Marginal sinus of the mediastinal lymph node with aggregates of diesel particles entering from the afferent subcapsular lymphatic vessels.

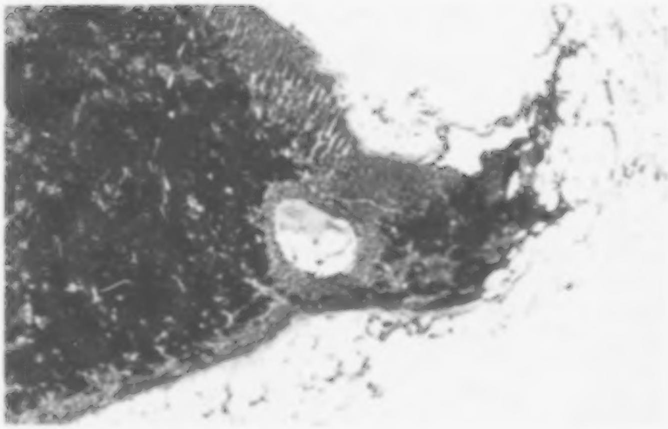

Figure 16 Lower power photomicrograph of a bronchial hilus lymph node with masses of diesel particles sequestered in the parenchyma (guinea pig exposed to 1500 $\mu g/m^3$ for 35 weeks). x 50

increasing accumulation towards the lymph node hilus (Figures 16,17).

It is, therefore, not surprising that with the continuing exposures, a distinct discoloration of the regional lymph nodes was observed at early stages of exposure and a solid black color of lymph nodes in the pulmonary hilus and mediastinum by the culmination of the exposure period (Figure 18).

Since similar accumulation of pigmented lymph nodes routinely is observed in the smoking population and in populations exposed to other forms of dust without further pathological changes in the lymphatic tissue, we feel that this phenomenon should be evaluated as a positive indication of the defense of the organism against the inhaled aerosol and considered more as a sign of excessive exposure, than as evidence of the biological activity of the inhaled diesel particulates. Continuing studies are certainly needed to assess any potential effects of the deposited pigment on the immunological function of the lymphatic system and to evaluate the impact of deposited particulates on the cellular populations released from the lymph nodes in to the general circulation. The present investigation is exploring this clearance mechanism and, although much work still needs to be done, our observations to date indicate that the lymph nodes have a large capacity to sequester diesel particulates deposited in the respiratory system preventing their influx into the general circulation. The nodes in turn, although stuffed with diesel particles, show no other pathological alterations.

It is, therefore, concluded that by this sequestration, the lymphatic system plays an important role in the clearance of inhaled diesel particles from the lungs and may be expected to provide significant protection in addition to mucociliary clearance against the effects of the inhaled diesel particulates. Both systems have a large capacity to remove inhaled diesel particles from the sensitive cellular structures of the respiratory system and seem to be fully capable of protecting the general population against any potential adverse health effects of diesel emissions, particularly under the conditions of low level exposures expected on our roads with the expanded use of diesel engines.

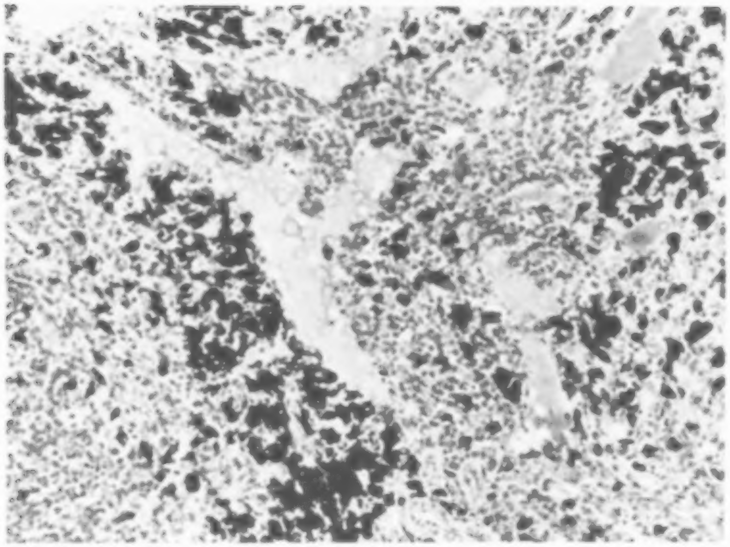

Figure 17 Higher magnification showing the accumulation of diesel particles in the perisinusoidal region of a mediastinal lymph node (guinea pig exposed to 1500 μg/m³ for 35 weeks). x 140

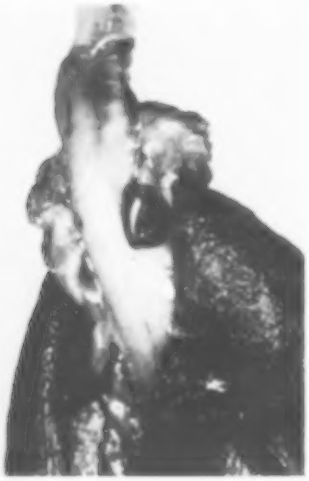

Figure 18 Discolored mediastinal node in a guinea pig exposed to 1500 μg/m³ for 35 weeks.

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General Discussion

P. NETTESHEIM: I am quite surprised that you haven't made an attempt to look for the early alterations in the lung. You can't, of course, do that by making a nice, logical picture and hoping to see something. You would have to do something . little bit more elaborate like, for example, doing some morphometic studies. I think that is a good occasion to do that because you are obviously loading your animals tremendously. The other comment I would like to make is a suggestion to you and maybe some of the others in the audience, since you do have labeled particles which you could use as tracers, couldn't one use this sort of technique now to determine under real conditions what the bioavailability of the substances associated with the particles are? Since you can measure the clearance of the particles from the lung, you should also be able to measure how the material is disassociated from those particles as they are residing in the lung. It would take a big experiment with maybe 100 rats, massively exposed for a relatively short time, but I think it could protentially yield some enormously valuable data.

J. VOSTAL: I think we will find the response to your first comment about the morphometry in the next presentation given by Professor Barnhart from the Wayne State University. As far as the other is concerned, we are having big hopes in the reductive way of the diesel particulates, but we have to say that we have not yet been completely over the river. We still have a problem in that over a longer time the label doesn't seem to stay with the particle and this might still be a problem. We don't want to go any further beyond the deposition rate, which is the determination you have immediately after inhalation, unless we could be assured that the lable is fixed to the particle and indicates to us the movement of the particles rather than the movement only of the lable.

IMPACT OF DIESEL ENGINE EXHAUST (DEE) PARTICLES ON THE STRUCTURAL PHYSIOLOGY OF THE LUNG

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ABSTRACT

This is a progress report on a systematic (scanning, transmission and light) microscopic and functional study of rat and guinea pig lungs exposed to short term (2 wk) and long term (to 1 yr) influences of DEE. Major attention is directed at detection of changes in the distal lung. Ultrastructural data is generated from coded specimens following a preestablished schedule on random tissue specimens observed and photographed randomly at low (X 1000 to 4000) and high (> X 5000) magnifications. The data base (micrographs) is analyzed by methods of quantitative morphometry. Several unusual findings are notable although the distal lung has not been extensively compromised during sub-acute or chronic exposures of 2 weeks to 3 months at DEE levels of 250 μg, 1500 μg and 6000 μg/M^3 in the ambient air. 1) DEE particulates are evident within pulmonary alveolar macrophages which increase in number and accumulate at bronchoalveolar junctions. 2) Epithelial type 1 cells (from the 2 week 6000 μg and the 3 month 250 μg and 1500 μg groups) occasionally display focal internal accumulations of DEE particles. 3) Epithelial type 2 cells increase in number in animals exposed to 1500 μg/M^3. 4) Granulocytic leukocytes, especially eosinophils, emigrate early in response to the higher doses of DEE-P in guinea pigs. However in that species, neutrophils typifying acute inflammation are rare in the lavage specimens.

INTRODUCTION

The impact of inhalation of diesel engine exhaust particulates (DEE-P) is being assessed in short term and chronic experiments in two animal species (rats and guinea pigs) selected for study because they have different sensitivities to toxic agents. In these studies the defense mechanisms are intact, initially at least, and are able to modulate the impact of DEE-P on the pulmonary cell types that are responsible for the structural physiology of the lung. The experimental design makes heavy use of systematic ultrastructural evaluations of the lungs exposed through natural inhalation of several different DEE-P concentrations (namely 250, 1500 and 6000 $\mu g/M^3$ of ambient air which corresponds to 1:240, 1:40 and 1:10 dilutions of total exhaust with ambient air). Diesel engine exhaust contains fine carbon particles, low levels of certain gases (CO, NO, NO_2,SO_2) and small concentrations of hydrocarbons (1). The health impact on intact animals is essentially unknown at the present time.

Our major attention is directed at detection of changes in the distal lung. We are examining, also in some detail, the effectiveness of phagocytic defense by pulmonary alveolar macrophages (PAM) in both in situ and in lavaged specimens. Much of the ultrastructural data is generated from specimens that are coded after animal delivery to our laboratory from General Motors Biomedical Laboratory. The codes are later matched to exposure conditions. In this way bias is minimized and when coupled with the quantitative morphometry done on the specimens may permit concentration effects and/or tolerance to be revealed and placed in correct perspective. Thus we have a great deal of data not appropriate for presentation now because it is still encoded. Later on, however, we expect to document the sequential changes that may occur, any specific injury responses, any tolerance, any adaptation or repair responses and finally the exact magnitude of these changes as related to dose and duration of exposure to DEE-P.

The thrust of our program is to provide exact morphologic data that is also presented in a quantitative manner in accord with the principles suggested by Weibel (2) and Crapo (3). These investigators, as our group are concerned with avoiding subjective and perhaps ambigious information generated from simple observation of micrographs that admittedly provide a highly selected view of the lungs' ultrastructure.

There are two phases of the ongoing work that I want to report at this time. First to be discussed is a pilot study involving a two week exposure of Sprague-Dawley rats and Chs. Rivers' guinea pigs to ambient air of approximately 6000 µg DEE-P/M^3. The stress on defense mechanisms under these conditions is clearly evident as well as certain data suggesting that epithelial pneumocytes (Pn), types I and II, respond to such exposure. Second, limited details from broncholavage specimens of guinea pigs subjected to DEE-P in both short and long term exposures are now available and will be briefly discussed.

MATERIALS AND METHODS

ANIMALS AND EXPOSURE CONDITIONS

Sprague Dawley and Fischer rats 344 and Hartley guinea pigs (Charles River Breeding Laboratory) were used in this study which is a cooperative one with General Motors Biomedical Research Laboratory (GMBRL). Exposure of animals to either DEE-P or to "clean" air was done at GMBRL where monitoring of the air that was being inhaled also was done. All animals, individually caged, were exposed for 20 hr/day and 5.5 days/week; thus one exposure week represented 110.5 hrs. Exact conditions for this inhalation study at GMBRL are described by Shreck and associates (1). At the end of the planned exposure, 3 fasted animals of each species for each time interval were delivered by GMBRL to our laboratory at Wayne State University. Every animal was assigned a code number which converted the GMBRL animals into a series of animals with exposure specifications unknown to investigators who would subsequently do the morphology and morphometry.

Pilot Study

Nine Sprague Dawley rats and 5 Hartley guinea pigs were distributed among 5 study sets of animals kept for approximately 18 weeks prior to sacrifice. All animals were in quarantine for approximately 2 wk after purchase. Following this, 3 sets of rats and 2 sets of guinea pigs entered the following exposure regimen. Control, age matched, rats and guinea pigs breathed "clean" air for the 15 wks under study; their total exposure to particles (dust, food particles, animal dandruff etc. generated by the animals moving about in their cages) amounted to 0.0325×10^4 µg/M^3. days for rat controls and to 0.0261×10^4 µg/M^3. days for guinea pig controls. One set of 3 rats inhaled air containing 6000 µg DEE-P/M^3 for the final 2 wk of the 15 wk study period; their total exposure to DEE-P amounted to 5.15×10^4 µg/M^3. days. The third set of 3 rats and second set of 3 guinea pigs had a varied and intermittant exposure to DEE-P alternating with clean air throughout the 15 week exposure period. During this interval they were exposed first to 2 wk of 250 μg DEE-P/M^3, then were on clean air for 5 wk, then breathed 250 μg DEE-P/M^3 for 5 wk, rested for 1 wk on clean air and finally breathed 6000 μg DEE-P/M^3 for 2 wk. Calculation of the total exposure dose for these 2 sets from the monitor data on particulate concentration indicated that the rat set received 5.15 x 10^4 μg DEE-P/M^3. days, while the guinea pigs received 6.01 x 10^4 μg DEE-P/M^3.days.

Tracheobronchial Lavage Study

Nine Hartley guinea pigs comprise this study on chronic exposure for 8 mon to either 250 μg DEE-P/M^3 or 1500 μg DEE-P/M^3 and include a concurrent age matched control set of 3 animals. The calculated exposure to particles was 0.23 x 10^4 μg/M^3.days for the control set. Exposed sets of 3 animals each was 4.93 x 10^4 μg DEE-P/M^3.days for the low dose set and 29.2 x 10^4 μg DEE-P/M^3 for the intermediate dose set. Two guinea pigs exposed for 6 mon were lavaged.

PHYSICAL CHARACTERISTICS OF THE DEE-P

Well dispersed DEE-P was delivered to the inhalation chambers in an airflow of 2.8 L^3/min, which permitted a 3-15 second residence time in the chamber. The mass median aerodynamic diameter of the diesel exhaust aerosol was 0.19 ± 0.03 μm with 88 ± 5% of the mass in particles smaller than 1 μm according to measurements by Soderholm (4). Diffusion battery measurements showed the mass median mechanical mobility diameter was between 0.10 and 0.15 μm. Monitoring of the exposure chambers established that the average particulate mass concentrations were within 2% of the target dose values. Spatial distribution measurements showed that the particulate mass concentration throughout the chamber and cage racks was uniform within 10%.

RETRIEVAL AND CHARACTERIZATION OF CELLS IN TRACHEOBRONCHIAL AIRWAYS AND ALVEOLAR AIRSPACES

Anesthetized animals were broncholavaged through a plastic cannula (scalp vein set) with an attached 19 guage needle using 5 repeated aliquots of 8-10 ml of McCoys' 5A complete culture medium containing 20% fetal calf serum following the procedure of Myrvik et al (5). The washes were pooled centrifuged and the cells resuspended in 6 ml McCoys' medium. The total number of cells recovered was counted immediately in a hemocytometer. A total cell differential count was performed on Leishman stained smears or cytocentrifuge specimens to establish the percentage distribution and absolute number of identifiable cells retrieved by the lavage procedure. Cell sizes were established by measurements from light micrographs of glutaraldehyde fixed cells in suspension and viewed by Nomarski differential phase-contrast optics. A Zeiss MOP III Digital Image Analyzer was used for cell size distribution studies.

Functional Tests

PAM viability was judged by ability of the lavage cells to exclude Trypan blue (6).

Glass adherence ability, a known property of macrophages, monocytes and to a lesser extent granulocytic leukocytes (5), was used. Our procedure utilized aliquots of the lavage concentrate placed in an open ended glass well (I.D. = 0.6 cm, height = 1 cm) overlaying a glass coverslip contained within a petri dish. The preparation was incubated for 2 hr at 37°C in a 5% CO_2 incubator. Next coverslips were rinsed twice to remove non-adherent cells and fixed with 300 mOsm, 1% glutaraldehyde in cacodylate buffer, pH 7.4. The coverslips were randomly photographed by light microscopy (Nomarski optics) before critical point drying for correlative scanning electron microscopy.

Phagocytosis of latex particles was used to guage the functional abilities of lavage cells. Cultured PAM were fed with fluorescent latex (0.6 µm) microspheres and uptake observed with a Zeiss fluorescent microscope. That particles were inside phagocytic vacuoles of PAM was verified by transmission electron microscopy on thin sections.

ELECTRON MICROSCOPY (EM)

Both types of electron microscopy, scanning (SEM) and transmission (TEM) were done. Procedure for fixation and processing was somewhat different for the pilot study group and the chronic 8 mon. group that were broncholavaged.

Standard Procedure for Pilot Study Animals

Animals were anesthetized by an intraperitoneal dose of sodium pentobarbital (50 mg/ml). An incision was made in the trachea and a plastic cannula inserted. Cacodylate buffered 1% glutaraldehyde fixative was instilled under a fluid pressure of 20 cm H_2O to achieve fixation of the lungs under normal expansion. The trachea was tied off to retain the fixative within the lungs. The expanded lungs were removed from the thoracic cavity, weighed and placed in a specimen jar containing more glutaraldehyde fixative. The fixative in the jar was replaced the next day. After storage for several days at 4°C, cacodylate buffered 0.2M sucrose was used to rinse the lungs prior to dissecting them for SEM and TEM. The cardiac lobe was taken for the SEM study. Pieces (0.5 x 1 cm) were taken at random from the other 3 or 4 lobes of the lung for TEM study.

The TEM specimens were post fixed and stained by placing in 1% veronal acetate buffered osmium tetroxide solution. Following progressive dehydration with increasing concentrations of ethanol, the specimens were embedded in Maraglas. Semi-thin and ultrathin sections were obtained using a diamond knife on an ultramicrotome. The semi-thin sections were stained with toluidine blue, viewed and photographed with a Zeiss light microscope. The ultrathin sections were doubly stained with uranyl acetate and lead citrate. Examination and photography was performed with a RCA-EMU4 microscope operated at 50 KV.

The SEM specimens were progressively dehydrated in rising concentrations of ethanol. Critical point drying was achieved after transfer through Freon 113 into Freon 13. Specimens were mounted on metal stubs and gold sputtered before examination and photography in an ETEC Autoscan electron microscope operated at 20 KV.

Standard Procedure for Broncholavaged Animals

Following the broncholavage procedure to retrieve the relatively free cells from airways and alveolar airspaces, the still in situ lungs were fixed by tracheal instillation of glutaraldehyde and further handled according to that just described for both TEM and SEM specimens.

The broncholavage concentrates fixed in glutaraldehyde were pelleted prior to each reimmersions in buffered OsO_4, in serial ethanol solutions, infiltration with propylene oxide, and final embeddment in Epon 812 or poly/Bed 812 (Polysciences Inc.). Cultured PAM were handled in their monolayer state and after polymerization the monolayer was quickly separated from its glass surface using liquid nitrogen and glued to an empty beam capsule for thin sections. The "en face" thin sections were doubly stained with uranyl acetate and lead citrate for examination in the RCA microscope. Cytocentrifuge specimens or 2 hr. culture specimens were processed for SEM as previously described.

Morphometry Procedures

Micrographs were taken at random from both SEM and TEM specimens. The routine was to photograph SEM specimens at magnifications of X 1000 and X 4000 while TEM sections were collected from 3 separate blocks and randomly photographed at the lowest magnification for sharp focus, namely near X 1400 on our microscopes, and also, at higher magnifications X 4000 and X 8000. Photographic enlargement created a data bank for each animal with fixed magnifications for SEM at X 1500 and X 5000 while TEM micrographs were at X 3500, X 10000 and X 20000.

For SEM evaluations a Digital Image Analyzer (Zeiss) was used to trace the cell outlines or total surface area of each micrograph. It is recognized that exact measurements can not be obtained from such treatment of the 3-dimensional SEM micrographs when they are treated as 2-dimensional flat regions. However, we believe the approximation of maximal diameter and relative surface area provide useful data for group comparisons.

For the morphometry of TEM specimens we used a plastic overlay consisting of 1 cm lines with 1 cm between lines that were offset 0.5 cm to provide an unbalanced set of 300 points to count. In general but not exactly we followed procedures advocated by Weibel (2) Burri et al (7), and Crapo and associates (3). The lung was treated as if it were homogeneous throughout. However, we believe this is unlikely at least with regard to the responses to DEE-P and think additional work should be done to determine if there are regions such as bronchiolar-alveolar and bronchoalveolar that should also be treated morphometrically. Morphometry has been done only with the X 3500 final magnification since we found fair agreement of our values with those already published (3). Certain detail of the lung, of course, cannot be quantified accurately without some point counting at higher magnifications (2,3). For each specimen a minimum of 5000 points where recognizable structures or air space intersected were counted (Table 1).

RESULTS AND DISCUSSION

PILOT STUDY ON SPRAGUE DAWLEY RATS

SEM permits an extensive overview of the 3-dimensional organization of the lungs and shows clearly at low magnification (X 50) the subdivisions into parenchymal (alveolar) and non-parenchymal areas. Through use of a digital image analyser on such SEM micrographs, taken at random, one can

TABLE 1. COMPARISON OF DATA OBTAINED FROM DEE-P AND CONTROL RATS WITH PREVIOUSLY PUBLISHED CONTROL RESULTS

PARAMETERS	Burri et al (7)	Weibel & Gil (8)	Crapo et al (3)	OUR PILOT EXPERIMENT Controls	Exposed Set 1	Exposed Set 2
AGE (DAYS)	131		77	150	150	150
BODY WT, (g)	457		360	517	501	503
LUNG VOL cm^3	13.37		10.70	13.96[a]	13.64[a]	13.70[a]
PARENCHYMAL (ALVEOLAR) VOL (%)			81	79.1[b]	81.0[b]	78.4[b]
FRACTIONAL VOL (%)[c]						
PAM		3.9	2.10[d]	1.92	6.42	7.66
Pn-I		13.0	11.96[d]	12.15	14.92	12.06
Pn-II		9.2	3.50[d]	3.74	6.57	5.80
ENDOTHELIUM		26.6	12.10[d]	12.83	11.80	13.11
INTERSTITIUM		47.2	21.10[d]	15.88	17.69	15.94
CAPILLARIES			61.30[d]	56.81	54.57	57.70
AIR			85.26[d]	67.94	70.16	69.20

[a] Calculated using formula ($V_L = 0.176 \times W^{0.70}$) from Burri et al (7).

[b] Calculated from SEM micrographs excluding the non-parenchymal structures (air ways and vessels larger than 25 μm).

[c] Calculated from point counting using overlay plastic grids of 224 points, Crapo (3) or 300 points for our data.

[d] Recalculated from raw data of Crapo et al (3).

quantify areas and volumes of the compartments and calculate the volume fractions (Table 1). For the rat control group the relative parenchymal percent was 79.1% while the exposed sets averaged 81%. These values agree well with those cited in the literature (3). The values for controls were not significantly different from the two-week 6000 μg/M^3 exposure groups which ranged between 78.4 to 82.1%. Thus during this time interval of 15 weeks of varied exposure to DEE-P there appeared to be no significant difference in the relative amounts of parenchymal tissue among the 3 groups of rats. This method of evaluation of course cannot be expected to detect changes in the relative percent of the various cell types that comprise the lung substructure.

Therefore, using TEM micrographs taken at random and photographically enlarged to magnification X 3500, point counting was done to determine the volume fractions (%) for four cell types (PAM, Pn-I, Pn-II and endothelial cells) and for the capillary, interstitial and air compartments of the alveolar lung (Table 1). There is good agreement between our rat controls and those of Crapo and associates (3) although we apparently did not expand the lungs to as great an extent and the morphometry was done somewhat differently. Some of the morphometry data of Weibel and Gil differs (8). The two significant differences noted upon exposure to the 6000 μg DEE-P/M^3 dose was the 4 fold increase in the fractional volume percent of PAM and a 1.75 fold increase of Pn-II over control values.

Changes in Pulmonary Alveolar Macrophages (PAM)

The sparcity of PAM (near 2%) in control rats was substantiated by the SEM observations (Table 2). About 4 control alveoli were patrolled by a single PAM (Fig. 1) while about every alveolus had one or more PAM following the exposure to the 6000 μg DEE-P/M^3 dose (Fig. 2). The change in PAM number/alveolus amounted to a 2.6 fold increase. Surface area measurements were done on the SEM micrographs and revealed that an average control PAM covered only 51.3 μm^2 of alveolar surface while an exposed PAM covered a 2.6 fold greater surface area, namely 133 μm^2. Notable was the irregular distribution of PAM among alveoli.

Uptake of DEE-P by PAM

The remarkable uptake of DEE-P by the PAM was clearly seen by TEM (Fig. 3). Individual features of phagocytized DEE-P were revealed at high magnifications where the particles measured from 0.014 to 0.072 μm. There were 2 types of electron dense particles contained within the

TABLE 2. IMPACT OF DEE-P INHALATION ON RATS IN 2 WEEK PILOT STUDY, 6000 $\mu g/M^3$: IN SITU PULMONARY ALVEOLAR MACROPHAGES (PAM)

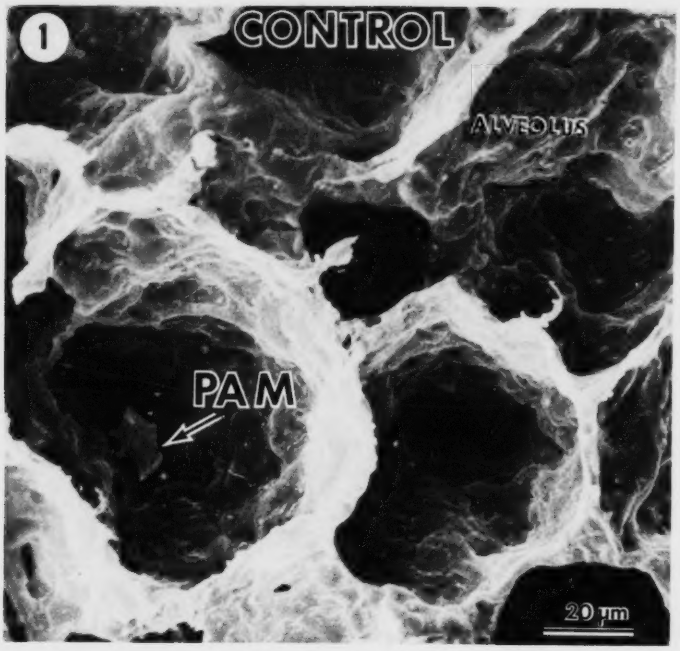
PARAMETER	CONTROLS	EXPERIMENTAL	SIGNIFICANCE LEVEL
PAM NO.	36	82	
ALV. NO.	127	110	
PAM/ALV.	0.28	0.74	$p < 0.15$ NS
PAM AREA (μM^2)	2157.5	4131.5	$p < 0.01$ S
ALV. AREA (μM^2)	287563.4	211898.2	
PAM SURFACE PATROL PAM-A/ALV-A	51.29	133.28	$p < 0.015$ S

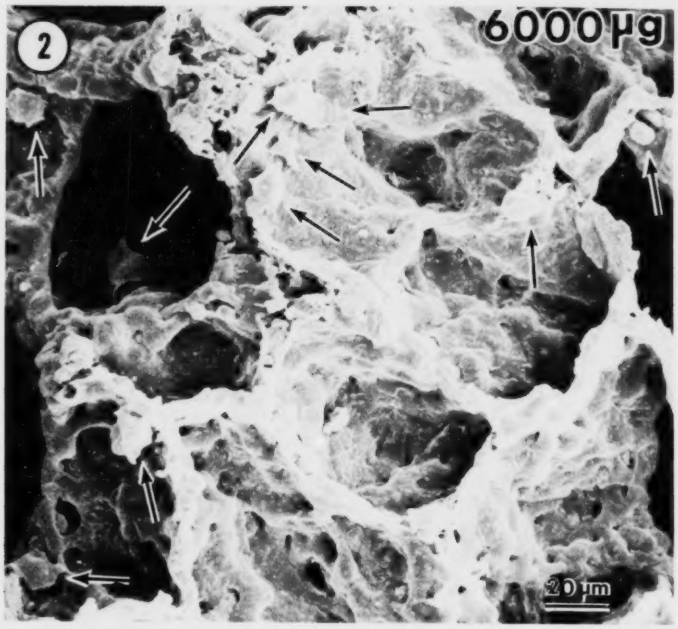
A = AREA; ALV. = ALVEOLUS; NS = NOT SIGNIFICANT; S = SIGNIFICANT BY STUDENTS "T" TEST; 3 RATS/SET

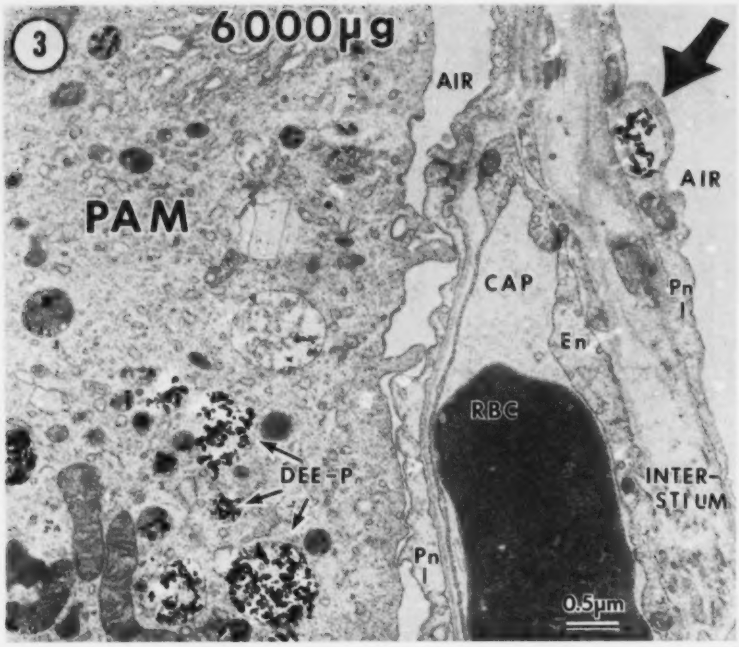
TABLE 3. COMPARISON OF ULTRASTRUCTURE OF LAVAGED GUINEA PIG LUNGS EXPOSED TO 250 μg DEE-P FOR SIX MONTHS WITH NON-LAVAGED AGE MATCHED SIMILARLY EXPOSED AND CONTROL LUNGS

PARAMETER	PERCENTUAL VOLUME DISTRIBUTION		
	EXPOSED LAVAGED LUNGS	EXPOSED NON-LAVAGED LUNGS	NON-EXPOSED CONTROL LUNGS
PAM	20.86%	17.76%	6.09%
Pn I	9.59	17.13	14.83
Pn II	13.05	10.59	7.42
ENDOTHELIAL CELLS	13.78	16.20	13.10
INTERSTITIUM	19.83	22.43	18.05
CAPILLARIES	36.68	32.09	54.76
AIR	63.00	72.59	72.32

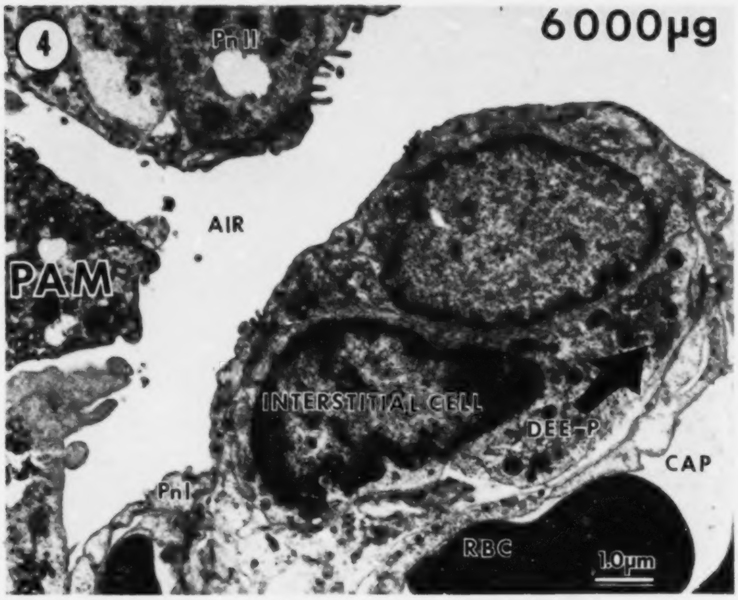
TWO ANIMALS/SET


1
CONTROL
ALVEOLUS
PAM
20 μm


2
6000 μg
20 μm



③
6000µg
AIR
AIR
PAM
CAP
Pn I
En
RBC
DEE-P
INTERSTITIUM
Pn I
0.5µm



④
Pn II
6000µg
AIR
PAM
INTERSTITIAL CELL
DEE-P
CAP
Pn I
RBC
1.0µm

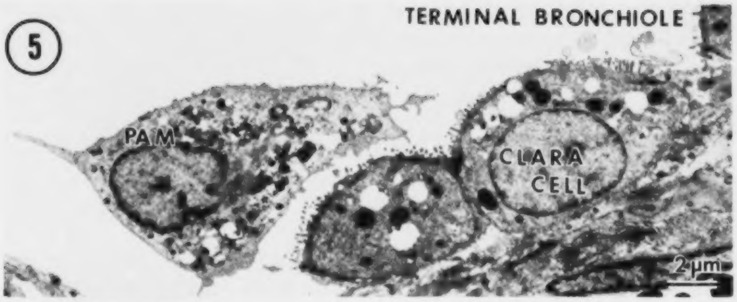
phagolysosomes which although irregular had maximal diameters measuring between 0.67-1.45 μm. The smaller particles had an average diameter of 0.021 μm and consisted of a dense core that was grey rimmed. The larger particles were very dense (black) and had an average diameter of 0.05 μm. Both types of particles were found together within the phagolysosomes.

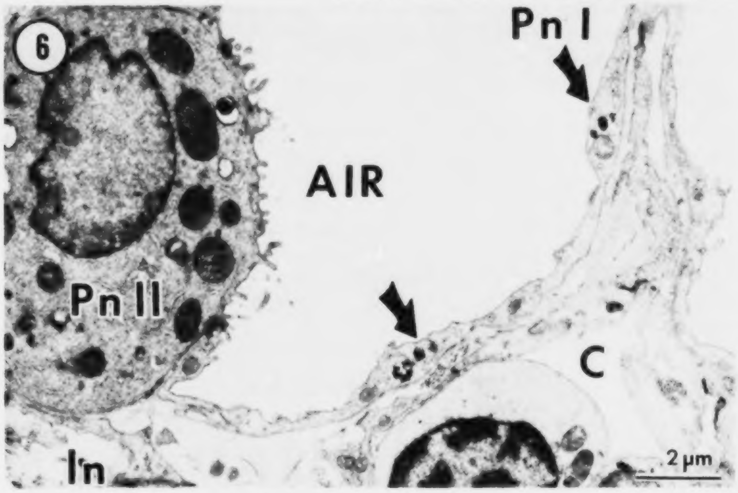
Translocations of DEE-P

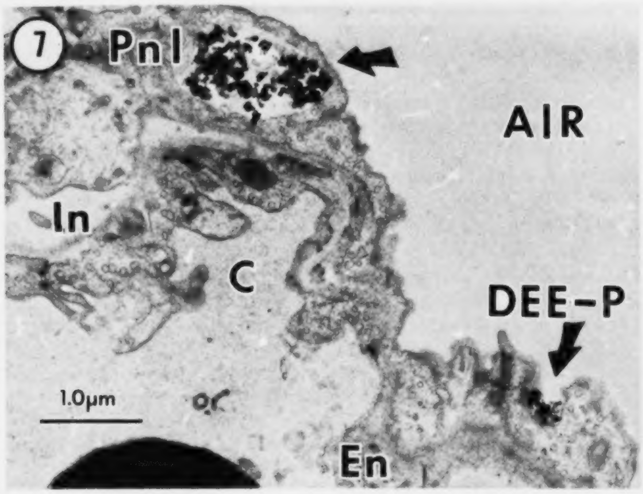
DEE-P appeared to stay enclosed within membrane bound structures within cells rather than free in the cytoplasm or on the basement membrane. However, some interstitial cells, presumably macrophages, contained DEE-P (Fig. 4). This interstitial location near the bronchiolar-alveolar junction probably illustrates part of the translocation of the DEE-P to lympho-hemotogenous drainage sites. PAM loaded with DEE-P were seen within lymph vessels in this region. However, it is also possible that the interstitial cells that have taken up DEE-P are quite young PAM or the immediate precursor of PAM (9). Also, DEE-P filled PAM were noticed on the epithelium of the terminal bronchiole (Fig. 5) evidently preparing to exit the lung by way of the major clearance mechanism, the mucociliary esculator (10). Much work needs to be done to clarify how DEE-P is translocated within the lung.

Uptake of DEE-P by Pneumocytes I (Pn-I)

Another cell type is also capable of incorporating DEE-P under such stress, and that is an epithelial cell, the Pn-I. The distinctively sized DEE-P was in vesicles within Pn-I, thus the normal epithelial cell barrier was breached (Fig. 6). How does this come about? Certainly phagocytosis is one possible means for DEE-P to penetrate or be taken into the Pn-I and Fig. 7 would be compatible with that interpretation. However, it is more likely that the DEE-P was pinocytosed and vesicles fused to give a larger vesicle. It is also possible that DEE-P could penetrate or enter at weakened cell junctions between adjacent epithelial cells which normally having tight junctions. Neither SEM nor thin section analysis of the alveolar epithelium from the pilot study animals have revealed separated junctions between the DEE-P stressed epithelial cells. Quantitative morphometry did not reveal any significant change in the fractional volume percent of Pn-I between the 2 exposed sets and controls (Table 1). However, the quantitative morphometry on the still encoded chronic study animals is expected to be more instructive on this point.


5
TERMINAL BRONCHIOLE
PAM
CLARA CELL
2 µm


6
Pn I
AIR
Pn II
C
In
2 µm


7
Pn I
AIR
In
C
DEE-P
1.0µm
En

Effects of DEE-P on Pneumocytes II (Pn-II)

Although these cells were prominent in specimens examined by SEM and TEM any change in their fractional volume percent during exposure to DEE-P is modest and not statistically significant (Table 1).

PILOT STUDY ON GUINEA PIGS

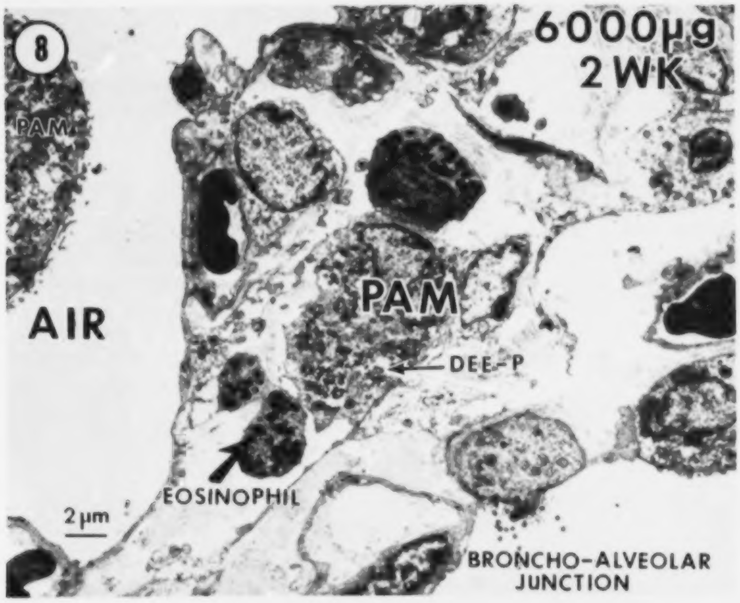
Results were very similar to those described in detail for the rat series. DEE-P was taken up by both PAM and Pn-I cells and appeared as discrete particles, average diameters near 0.05 μm, which were in phagolysosomes or other membrane enclosed vesicles. The unusual finding was the appearance of eosinophilic leukocytes and "reactive" monocytes in the alveolar airspaces. The eosinophils were especially prominent at bronchiolar-alveolar junctions where they were occasionally seen in the interstitium (Fig. 8).

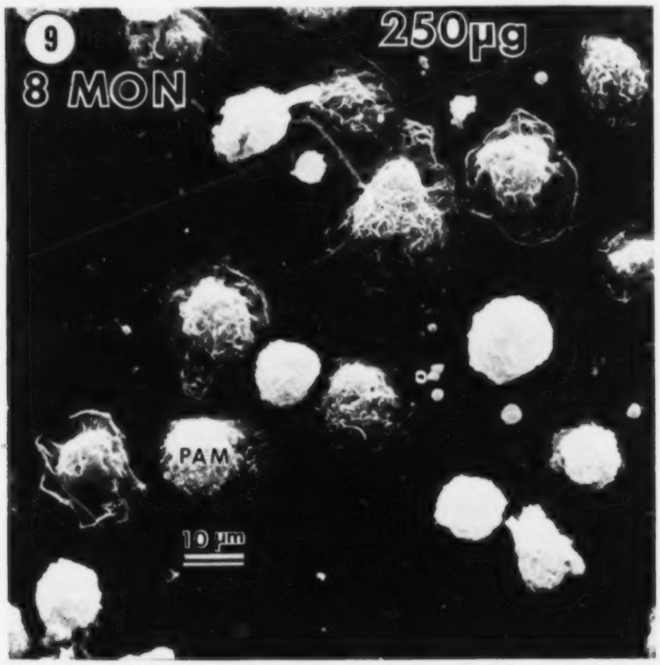
CHARACTERISTICS OF PAM OBTAINED BY BRONCHOLAVAGE OF GUINEA PIGS EXPOSED FOR 8 MONTHS TO DEE-P

Cells were obtained by tracheobronchial lavage from 9 animals in a chronic exposure group which included 3 controls. The exposure group given the dose of 250 μg DEE-P/M^3 actually received a total exposure during the 8 mon. study of 5×10^4 μg/M^3.days. This total exposure is near to the total exposure dose received by the short term exposure guinea pigs in the pilot study which value was 6×10^4 μg DEE-P/M^3.days. However, the 8 mon. animals exposed to 1500 μg DEE-P/M^3 received a much larger total exposure, namely 30×10^4 μg DEE-P/M^3.days.

Viability of PAM

The viability studies showed that 90-97% of the PAM excluded Trypan blue. There was not a significant difference in viability between either exposed groups (92-97%) and controls (90-97%). Our results with in vivo exposure of the lung PAM to DEE-P are in direct contrast to those reported by Aranyi and associates who demonstrated PAM cytotoxicity upon in vitro exposure of lavaged cells to fly ash (11). They demonstrated that the smaller particles < 2 μm were the most toxic. Although toxic minerals and hydrocarbons were possibly bonded, adsorbed or carried by the fly ash particles, generated from coal burners operated at high temperature, they were unable to attribute the toxicity of PAM to leaching minerals. In their experiment PAM viability was decreased to 30-80% and related to the size


8
6000µg
2 WK
PAM
PAM
AIR
DEE-P
EOSINOPHIL
2 µm
BRONCHO-ALVEOLAR JUNCTION


9
250µg
8 MON
PAM
10 µm

of the fly ash particle.

Phagocytosis Ability

PAM from all experimental sets phagocytized fluorescent microspheres (0.6 μm) as judged by fluorescence microscopy. Quantitative differences in phagocytic ability may exist since no attempt was made to quantify these data. That the exposed PAM had actually phagocytized latex was confirmed by TEM which revealed latex particles within phagosomes some of which also contained DEE-P.

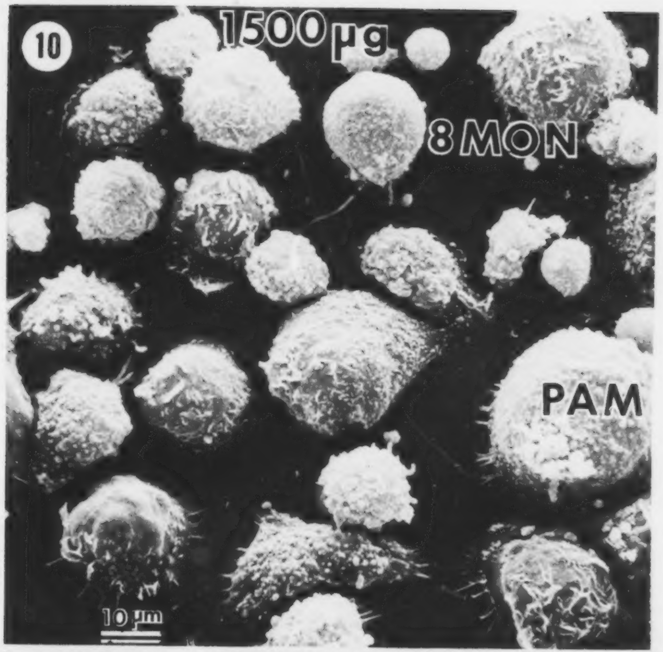
Enhanced Adhesiveness and Spreading Ability of DEE-P Exposed PAM

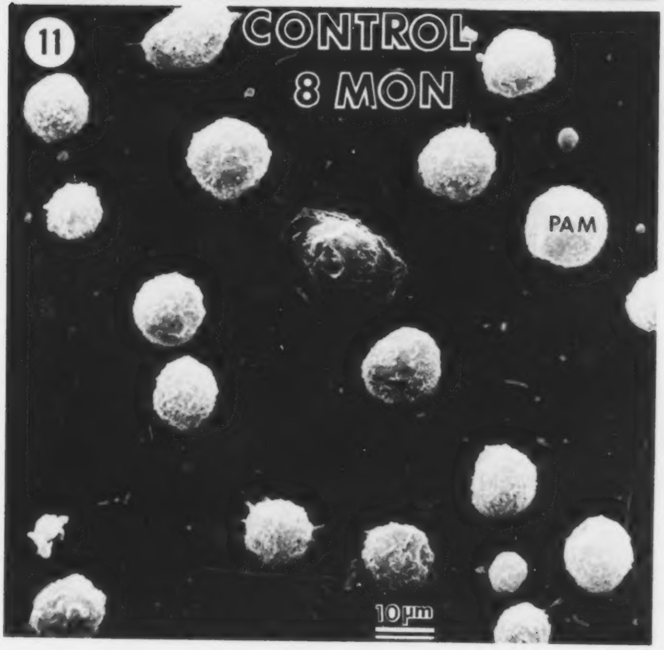
The ability of exposed PAM to adhere to and spread on glass and plastic coverslips during a 2 hr period under culture conditions was more pronounced (Figs. 9,10) than that of control PAM (Fig. 11). Furthermore these greater abilities of exposed PAM were dose related. Much more work is needed here to determine if the enhanced spreading ability is a signal that the PAM responding to the stress of DEE-P are more activated than control PAM. The possibility also exists that PAM are a heterogenous population with respect to functions (10).

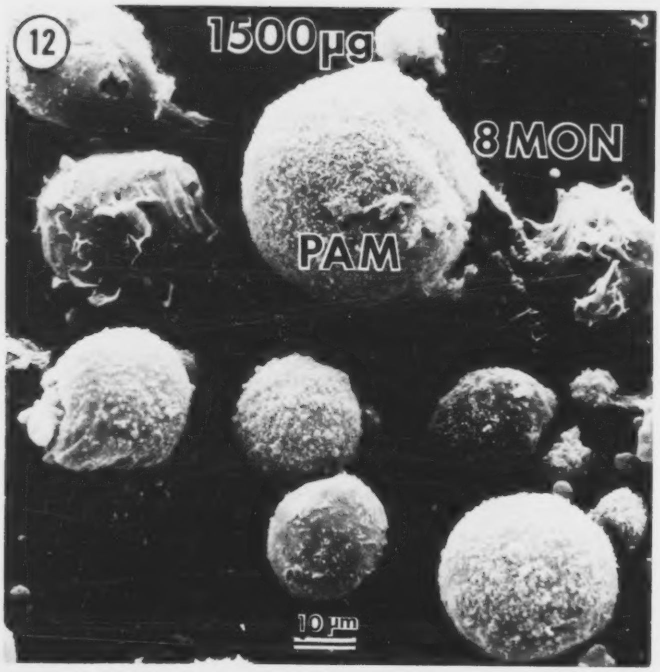
Size and Surface Membrane Variations

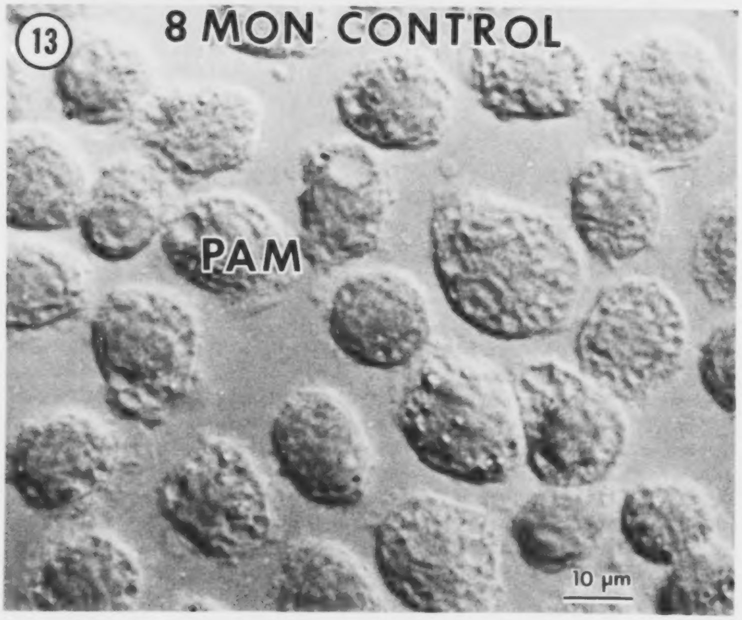
A notable finding was the tremendous size variation among the cells in the lavage specimens and also in the adherent cells which was particularly prominent at the 1500 μg/M^3 exposure dose (Fig. 12). SEM micrographs of adherent PAM from the control, 250 μg DEE-P and 1500 μg DEE-P sets were measured with a digital image analyzer to obtain maximal diameters and relative surface areas. The control PAM had an average diameter of 13 μm (range 9-14) in contrast to the 250 μg DEE-P cells which measured 17 μm (range 10-24) and the 1500 μg DEE-P cells which had an average diameter of 25 μm (range 10-44). The relative surface areas for PAM control were 120 μm^2 (range 70-160) vs 209 μm^2 (range 100-300) vs 394 μm^2 (70-1400) for the respective exposed sets. Thus a 2 fold increase in diameter and a 3 fold increase in surface area occurred in the PAM exposed for 8 mon. to the 1500 μg DEE-P/M^3 dose.

Surface activity of the exposed PAM resembled that of controls (Fig. 11) except that it often was more extreme. Ruffling was especially prominent in the 250 μg DEE-P/M^3 set (Fig. 9). Blebs were notable in more cells of the 1500 μg DEE-P/M^3 set (Fig. 10) than in controls. Increased numbers of cells with partial or completely smooth


10
1500 µg
8 MON
PAM
10 µm


11
CONTROL
8 MON
PAM
10 µm


⑫
1500µg
8 MON
PAM
10 µm


⑬
8 MON CONTROL
PAM
10 µm

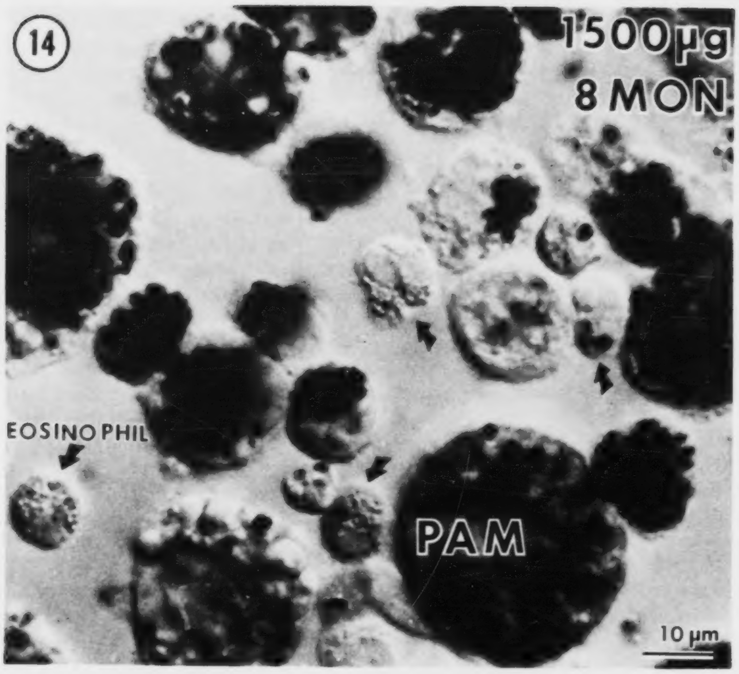
surfaces appeared in the highest exposure PAM (Fig. 12). It is quite probable that application of quantitative morphometry to cells adhering in this functional test for one of the fundamental properties of PAM will reveal significant dose related differences (12).

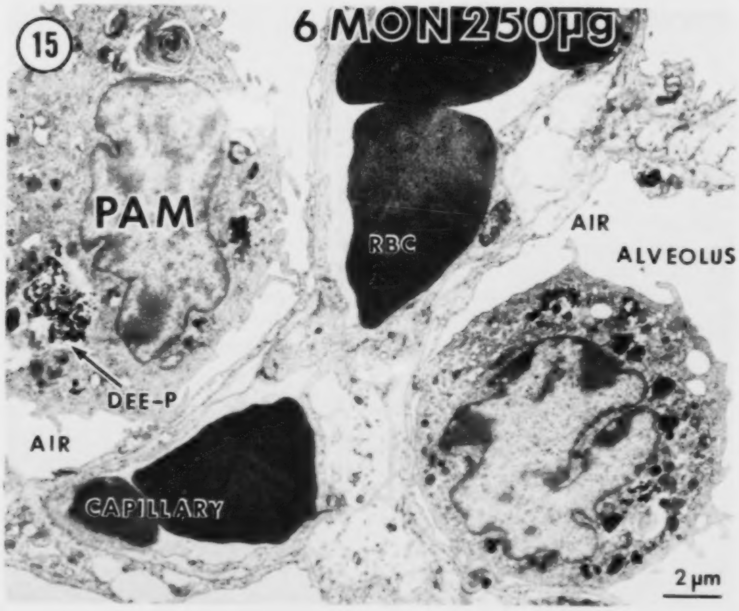
Identification of a Small Cell Population in the In Vivo DEE-P Exposed Lavage Specimens

Because of the great size variation among cells in the lavage specimens of exposed animals, it became important to consider that some other cell types than PAM might be present. Some insight was gained from light microscopy with Nomarski differential interference contrast optics which displayed the unstained adherent cells. From the control lavage essentially all of the cells were PAM (Fig. 13). It was apparent in the 1500 μg DEE-P exposure group that most of the cells regardless of size contained the DEE-P; these were whole cells and consequently one cannot distinguish individual DEE-P but sees instead the black phagolysosomes (Fig. 14). It became apparent that the small cell type was granulocytes, namely eosinophilic leukocytes whose granules, approximately 0.5 μm in size, are distinctive in Nomarski microscopy. Moreover examination of Leishman stained smears confirmed that eosinophils were in the lavage specimens. Differential counts showed that 26% eosinophils were in the 1500 μg DEE-P set, 14% eosinophils were in the 250 μg DEE-P set to contrast with 3% in control lavages. Phagocytosis of DEE-P by the eosinophils was frequent in exposed animals. However, the blood smears made from cardiac punctures of these animals did not show a similar order of eosinophilia. Neutrophils, which one would expect to be present if this was an acute inflammation, were rare in any of the guinea pig lavages. It is well known for the guinea pig that eosinophils are found in increasing numbers in chronic reactions (13).

Recognition of "Reactive" Monocytes or Macrophages in the In Vivo DEE-P Exposed Specimens

Quite prominent especially in the 1500 μg DEE-P exposure group were a unusual group of large cells that were classified in the mononuclear PAM category. These cells that comprised about 20% of the lavage cell population exclusive of contaminating erythrocytes had multilobulated nuclei. By appearance in the Leishman stained smears and cytocentrifuged preparations, these bizarre cells resembled most the "reactive" monocytes notable in blood during certain chronic infections (14). However, we did not observe such cells in blood smears of exposed guinea pigs that had such "reactive" cells in their tracheobronchial lavages. Exami-


⑭
1500µg
8 MON
EOSINOPHIL
PAM
10 µm


⑮
6 MON 250µg
PAM
RBC
AIR
ALVEOLUS
DEE-P
AIR
CAPILLARY
2 µm

nation of lavages from other chronic DEE-P exposed animals should be instructive in indicating at what times and DEE-P doses these "reactive" monocytes or PAM appear.

PAM STILL IN SITU AFTER BRONCHOLAVAGE

The question of how representative of the total PAM population of the lung is the lavage specimen must be addressed. To gain data on whether or not all or most PAM were washed out of the lung we used guinea pigs exposed for 6 mon. to 250 μg DEE-P/M^3. Two animals were lavaged and then the lung was fixed in situ by instillation of the glutaraldehyde solution. Upon examination of these lungs it was quite evident that many PAM remained still adherent to alveolar walls (Fig. 15). Their number as well as other ultrastructural information was obtained by point counting morphometry and compared with lungs of companion exposed guinea pigs and age matched controls that had not been lavaged (Table 3). Admittedly our tracheobronchial lavage procedure is intentionally as gentle as possible and we do not massage the lungs. Thus with out procedure we only obtain a fraction of the PAM population of the lung. It seems likely that the lavage PAM thus represent those PAM that were already on the mucociliary esculator and possibly those PAM free in alveolar airspaces or loosely attached to alveolar walls. PAM that were not retrieved had taken up DEE-P but appeared still to be vacuum cleaning the alveolar epithelium. Of course PAM within the interstitium are not available to lavage fluids so they remain in place.

These data should strengthen an investigator's conviction that studies designed for determining in vivo effects of possibly toxic agents on PAM should investigate both in situ and easily retrieved PAM populations so that a more complete appreciation can be gained on the real effects of the agent(s). It is quite possible that most PAM obtained by lavage are the oldest, most debilitated cells of the population and are collected in the process of exiting the lung. However, it is still of considerable importance that lavage cells retain viability and show functional capabilities that are recognized for cells in relatively good health (10) even though burdened in our study with DEE-P in their phagosomes.

ACKNOWLEDGEMENT

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OVERVIEW OF THE HEALTH EFFECTS RESEARCH LABORATORY'S

(CINCINNATI) DIESEL EXHAUST HEALTH EFFECTS STUDY

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Cincinnati, Ohio 45268

ABSTRACT

The role of the Health Effects Research Laboratory (HERL) Cincinnati in evaluating the effects of exposure to diesel exhaust emissions is discussed. Also discussed briefly in order to achieve an overall perspective, are other research programs at HERL, Cincinnati as well as components of the Mobile Source Research Program being carried out at other EPA laboratories.

The primary mission of HERL, Cincicnnati in the area of diesel emissions research is to: 1) Evaluate the carcinogenic mutagenic and toxicological effects of exposure to diesel exhaust emissions via the inhalation route; 2) Determine the epidemiological effects of exposure to diesel exhaust emissions and; 3) Measure the incidence of tumor induction in Strain 'A' mice by diesel exhaust extract and other environmental pollutants via the intraperitoneal route. Individual projects within these areas are outlined.

INTRODUCTION

The mission of the EPA in the area of diesel emission research is to evaluate the possible hazards to human health resulting from exposure to diesel emissions, to characterize these emissions, to monitor diesel emission concentrations in ambient air, and to develop models for estimating concentrations of diesel exhaust components in ambient air. The development of control technology has generally been left to other agencies such as the Department of Transportation.

The diesel emission research being conducted by EPA is coordinated by the Mobile Source Research Committee. Membership on this committee includes representatives from participating laboratories, the regulatory offices, and EPA headquarters. The five laboratories involved in this program are listed below:

1. Health Effects Research Laboratory [HERL, Cin*]
2. Health Effects Research Laboratory [HERL, RTP**]
3. Environmental Monitoring and Support Laboratory [EMSL, RTP]
4. Environmental Sciences Research Laboratory [ESRL, RTP]
5. Industrial Environmental Research Laboratory [IERL, RTP]

* Cincinnati, Ohio **Research Triangle Park, North Carolina

The research being conducted at HERL, Cincinnati, comprises about 20% of the total EPA mobile source research program. On the other hand only 10% of the total research in progress at HERL, Cincinnati, involves diesel emission health effects studies. Other research programs being carried out in this laboratory include studies of the health effects of drinking water, water quality multiroute exposure, and energy development.

The two Health Effects Research Laboratories are the only ones directly involved with conducting health effects studies of engine emissions. The program at the two laboratories are complementary with very little duplication of effort. HERL-RTP, is primarily involved with in vitro testing of emission products from a variety of engines. The types of tests include the development and utilization of tiered test systems from Ames tests through mammalian cell cultures and the development and application of bioassay tests for gas phase organic samples. Certain whole animal tests are also being carried out such as skin painting, intratracheal instillation of diesel particulate, and evaluation of heritable effects.

The primary mission of HERL-Cincinnati, in the area of diesel emission research is to: 1) Evaluate the carcinogenic, mutagenic, and toxicological effects of exposure to diesel exhaust emissions via the inhalation route; 2) Measure the incidence of tumor induction in Strain 'A' mice by diesel exhaust extract and other environmental pollutants via the intraperitoneal route; and 3) Determine the epidemiological effects of exposure to diesel emissions. The remainder of this article will be devoted to a discussion of these three areas.

INHALATION EXPOSURES (WHOLE EXHAUST)

Exposure Conditions. Details of the exposure facility and exposure conditions have been presented elsewhere in the proceedings. Therefore, only a brief summary will be presented here. Long-term exposure studies have been in progress since September 1978.. Diesel exhaust is produced by a Nissan 6-cylinder diesel engine. The engine is operated on a dynamometer base plate at a varying speed and load corresponding to the "Federal Short Cycle." The engine is operated eight hours per day, seven days per week. The exhaust is diluted to achieve a particulate mass concentration as near 6 mg/m^3 as possible. As of November 19, 1979, the dilution ratio was altered to produce a particulate level of 12 mg/m^3. This mode of operation is planned to continue through 1980.

The animals are exposed in one of 24 stainless steel chambers of about 100 cubic feet volume. They are housed in wire cages with the exception of cats which are allowed to roam free in the chambers. Food and water are provided ad libitum.

Toxicology Studies. The diesel exhaust inhalation studies are broken down into two main groupings, non-oncogenic toxicology experiments and those emphasizing carcinogenesis and mutagenesis. The toxicology studies include pulmonary function and pathology, infectivity, behavior and neurophysiology, reproduction and teratology.

Pulmonary function measurements have been made on cats exposed for one year to diesel exhaust at a 6 mg/m^3 particulate level. These same cats are being exposed an additional year to 12 mg/m^3 particulate. Measurements have also been made on mice exposed three months and Chinese hamsters exposed six months at the lower exhaust concentration. Additional Chinese hamsters are being exposed six months to the higher concentration of exhaust.

Teratology studies using rats and rabbits were completed during the past year. Exposure to diesel exhaust during the critical period of gestation did not result in a detectable increase in birth defects. A multigeneration reproduction study using mice was begun during the fall of 1979. This study will be completed by the fall of 1980.

Behavioral and neurophysiological measurements were carried out during the past year using rats. Voluntary activity and learning were depressed. Nerve conduction was also altered in young animals. No studies are in progress at present. Resistance to infection was depressed during exposure to diesel exhaust. These studies have been completed and no additional studies are planned.

CARCINOGENESIS AND MUTAGENESIS

The experiments emphasizing carcinogenesis and mutagenesis are subdivided into direct and indirect measurements with an additional category comprising estimates of heritable mutations.

Direct Measurement of Tumor Induction. These include the following experiments:

a) Tumor incidence in Strain "A" mice
b) Tumor incidence in Sencar mice
c) Lung tumor induction in Syrian hamsters
d) Induction of liver islands in rats

The Strain "A" mouse model has been used in our experiments because of its demonstrated sensitivity to induction of lung tumors and the relatively short time period required for the development of tumors. Increases in tumor incidence were not detected in males exposed during 1979. Females did show a significant increase in lung tumors. These latter findings were questionable, however, because of a lower than normal tumor incidence in controls. Additional groups of mice are being exposed during 1980 at twice the exposure concentration of 6 mg/m^3 particulate used previously.

Another large inhalation exposure study is in progress utilizing Sencar mice. Since it is believed that this strain of mice has a lower incidence of spontaneous lung tumors than Strain "A" mice, longer exposures can be carried out. The sensitivity of the test animals will also be increased by exposing them from conception and by exposing them in the dark when they are more active, awake, and have a higher level of respiration. The prime objective of this study is to determine, should positive results be obtained, whether diesel exhaust acts primarily as a tumor promoter, initiator, or as a whole carcinogen. To test this hypothesis, groups will be exposed to diesel exhaust alone or concomitantly with butylated hydroxy tolulene (BHT), a lung tumor promoter or urethane, an initiator. Exposures were begun the summer of 1979 and will be completed by October 1980.

Exposure of Syrian Golden hamsters has been in progress since February 1979 and will continue until October 1980 for an approximate 20-month time period. Syrian hamsters were selected because of their low normal incidence of lung tumors, their resistance to respiratory disease, and the fact that the types of lung tumors developed by this species are more likely to resemble those found in humans than those normally found in mouse lungs.

The liver island test in rats has been utilized as a relatively short-term (three to six months) test for liver carcinogenesis. Partial hepatectomy plus a choline deficient diet will promote, that is, decrease the latent period for the appearance of preneoplastic lesions. The induction of gamma glutamyl transferease is used as a marker of carcinogen altered phenotype. Negative results were obtained from rats exposed three and six months during the past year. No further studies are in progress or are being planned.

Indirect Measurements of Carcinogenesis and Mutagenesis. The experiments in the category are listed below:

a) Hemoglobin adducts
b) Cytochrome P_{450} and AHH induction
c) Cytogenetics, micronucleus test, and urine mutagenicity in Swiss Webster mice
d) Sister chromatid exchange in Syrian hamster lung cells
e) Sister chromatid exchange, micronucleus test, cytogenetics, in Chinese hamster

The methodology for measuring hemoglobin BaP adducts is being perfected. This test is used as a dose monitor for the presence of carcinogens in the blood. Measurements will be made during 1980 using blood samples from rats and cats exposed chronically to diesel exhaust emissions.

Cytochrome P_{450} and AHH induction has been measured in Strain "A" mice exposed six months to diesel exhaust containing 6 mg/m^3 particulate. No important changes were detected. It is planned to repeat these measurements during the next year using animals exposed to 12 mg/m^3 particulate.

Cytogenetics, micronucleus testing and urine mutagenicity were measured during one, three, and seven days exposure of Swiss Webster mice. Results were negative. Only micronucleus testing will be repeated during 1980.

Sister chromatid exhange has been measured in Syrian Golden hamster lung cells. Positive results were obtained following intratracheal instillation of particulate but not after three months inhalation of exhaust. A second group is being exposed at the increased exhaust concentration of 12 mg/m^3 particulate. Sister chromatid exchange, cytogenetics, and micronuclei tests have been conducted using peripheral lumphocytes from Chinese hamsters exposed for three months. These measurements will also be repeated during 1980.

Heritable Mutations. Studies under this category are listed below:

a) Detection of heritable mutations in fruit flies
b) Detection of sperm head abnormalities in mice, hamsters, and cats
c) Detection of heritable defects in mice

During 1979 fruit flies were exposed for detection of heritable mutations. This study has been completed with negative results. Sperm samples have been collected from mice, cats and Chinese hamsters during the past year. Further sampling will be carried out during 1980 at the higher exposure level.

A large number of mice were exposed at our Center Hill facility for the detection of heritable defects. This study was coordinated by workers from EPA at RTP and carried out by Oak Ridge National Laboratories. Following exposure, the animals were transferred to Oak Ridge for breeding and evaluation. The types of endpoints included heritable point mutations, by the specific locus test, induction of dominant lethals in males and females, induction of heritable translocations, and oocyte loss. Results to date have not indicated detectable changes. No further exposures are planned.

INHALATION STUDIES (COMPONENTS OF EXHAUST)

The studies in this category are listed below:

a) Exposure to NO_2 and/or carbon particulates
b) Exposure to acrolein
c) Exposure to BaP and BaP plus carbon particulates

In the first study listed, the rats were exposed up to six months to either 2.5 ppm NO_2, 6 mg/m^3 carbon particulate, or both in combination. These levels were selected to coincide with NO_2 and particulate levels present in the diesel exhaust chambers during 1979. These studies have been completed and are reported elsewhere in the proceedings. Measurements included pulmonary function, pathology, behavioral parameters, and blood chemistry.

Rats were exposed up to 28 days to as much as 4 ppm acrolein for measurement of pulmonary function and pathology. This three carbon aldehyde with an additional double bond is one of the most irritating organic compounds present in diesel or gasoline engine exhaust. This study has been completed and the results are being analyzed.

Mice will be exposed to BaP and BaP plus carbon particulate to establish a dose-response curve for this carcinogen which is present in diesel exhaust. This study was planned in order to compare the effectiveness of diesel exhaust with that of a known carcinogen in inducing lung tumors. A secondary objective is to determine to what degree adsorption to a carbon particle increases the effectiveness of BaP in inducing lung tumors.

STRAIN "A" MOUSE INTRAPERITONEAL (IP) INJECTION

The Strain "A" Mouse IP Injection study is one of several experiments designed to compare the carcinogenicity of a variety of pollutants. Other tests in progress include in vitro tests, intratracheal instillation, and skin painting. The Strain "A" study is the only one being conducted at Cincinnati. The others are being conducted, or contracted, by HERL-RTP.

Because a variety of tests are being used to test several substances, the entire study has been called the matrix experiment. Other pollutants tested in the matrix include roofing tar, cigarette smoke condensate, coke oven emissions and possibly gasoline engine exhaust particulate extract. The comparisons are being made because of the availability of epidemiological data for the non-engine exhaust pollutants. By comparing the carcinogenicity of diesel emissions with pollutants such as coke oven emissions, of which considerable is known epidemiologically, it will be possible to estimate the cancer risk of diesel emission in humans.

Preliminary experiments to determine the maximum tolerated doses as well as to give some estimate of carcinogenicity are presently nearing completion. The principal portion of the IP study will begin early 1980. Completion is planned for 1981, although data for some of the pollutants will be available at an earlier date.

EPIDEMIOLOGY

Epidemiology work has proceeded somewhat slowly. Considerable effort has gone into attempts to define suitably exposed populations. During the past year, the feasibility of using various populations for further studies was completed. A critical review of diesel epidemiology was also finished. At present it appears that the best available population for a large scale epidemiology study are railroad workers. Records are available for a large number of these workers dating back to the beginning of dieselization in the United States during the 1950's. Depending upon availability of funds, such a study will begin during 1980.

SUMMARY

A wide variety of experiments are being carried out to evaluate the carcinogenic, mutagenic, and toxicological effects of inhaled diesel exhaust emissions. Although the concentrations used are far above ambient, extreme exposures are considered necessary to determine if diesel exhaust poses a carcinogenic risk to humans. It is hoped that this research, much of which was presented in detail during the symposium, will aid in providing a sound data base which will permit an accurate estimate of the health risk resulting from increasing number of diesel engine vehicles on the highway.

General Discussion

R. SCHRECK: Can you tell us something about the BaP and BaP plus carbon particle exposure? How is that going to be done? Is it going to be aerosolized? If so, what size?

W. PEPELKO: Actually, the details haven't been worked out, but we do plan on aerosolizing it and attaching the BaP to the carbon if possible. There have been some problems in attempting to do that. There is also a question of meeting safety requirements.

R. SCHRECK: What is the actual size of the particle?

W. PEPELKO: We plan to keep them about the same size as the diesel particles; less than a micron.

FACILITIES FOR DIESEL EXHAUST STUDIES

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ABSTRACT

It is the major intent of this report to examine the animal inhalation facilities at the EPA Health Effects Research Laboratory in Cincinnati, Ohio, associated with the exposure to the exhaust of diesel-powered equipment. The facility serves as a center for toxicologic evaluation, in relevant animal models, of potentially hazardous environmental pollutants from diesel engine exhaust. Briefly, the entire engine exhaust is mixed with filtered and conditioned air in a dilution tube. That diluted exhaust enters a large volume mixing chamber and a portion passes through dynamic flow irradiation chambers (to simulate sunlight) and is then conducted to animal exposure chambers. The system provides nonirradiated exhaust in the same concentration (directly from the mixing chamber) and also filtered, conditioned ambient air for control animal exposure. The major physical components of the diesel emission system include: air purifiers, animal exposure chambers, engine-dynamometers, irradiation chambers, monitoring instrumentation and data acquisition systems.

INTRODUCTION

The Environmental Protection Agency (EPA) is charged, under the Clean Air Act, with the responsibility for regulating emissions from new motor vehicles. It has been estimated that automobiles equipped with diesel engines are likely to capture up to 25 percent of the United States new car market by 1985(1). The concern over fuel economy is the major factor behind the projected increase, since diesel-powered vehicles offer approximately a 25 percent improvement in fuel savings over comparable vehicles powered by spark ignition gasoline engines. With the increase of diesel-powered automobiles, the anticipated change in the quantities of environmental pollutants emitted into the atmosphere indicates the need for well instrumented health effects studies to adequately establish their health impact.

Although diesel equipment is not widely used in American coal mines, it is extensively used in non-coal mines throughout the world. Its acceptance and use in underground coal mines has been very limited, principally because of concern for potential adverse health effects to miners exposed to the exhaust components. There is a substantial body of knowledge relating to coal mine dust and individual constituent pollutants in diesel exhaust, but there is very limited information on the biological effects of diesel exhaust in confined spaces such as underground mines. Diesel exhaust contains several components - including carbon particles, hydrocarbon compounds, and oxides of carbon and nitrogen which might reach concentrations that could pose a health hazard that may be anticipated due to the known incidence of respiratory disease among coal miners and the potential for interactions between respirable coal mine dust and diesel emissions. Pathologic changes from such combined exposures may differ from those resulting from exposure to either agent alone.

The National Institute for Occupational Safety and Health (NIOSH) is responsible for establishing air standards for coal mines and EPA for ambient air standards as affected by diesel-powered vehicles among others. Accordingly, the two organizations have signed an interagency agreement to conduct toxicological studies on animals exposed to combined diesel exhaust and coal dust. The EPA Center Hill facility serves as a center for toxicologic evaluation, in relevant animal models, of potentially hazardous environmental pollutants derived from the exhaust of a light duty diesel automobile engine and has recently been expanded to accommodate the interagency study.

FACILITIES

The System. Briefly, the entire engine exhaust is mixed with filtered and conditioned air in a dilution tube. That diluted exhaust enters a large volume mixing chamber and a portion passes through dynamic flow irradiation chambers (to simulate sunlight) and is then conducted to animal exposure chambers. The system provides nonirradiated exhaust in the same concentration (directly from the mixing chamber) and also filtered, conditioned ambient air for control animal exposure. There are two similar but separate systems for the automotive diesel exhaust and the mine diesel exhaust generation, since they require different engines and operating conditions. The principal difference is the absence of irradiation chambers and the added coal dust generators for certain exposure chambers in the mine diesel system. Figure 1 illustrates the flow and essential equipment in the automotive diesel emission system. The major physical components of the facility include air purifiers, engine-dynamometers, irradiation chambers, animal exposure chambers, monitoring instrumentation and data acquisition systems. This equipment is further described in the following text.

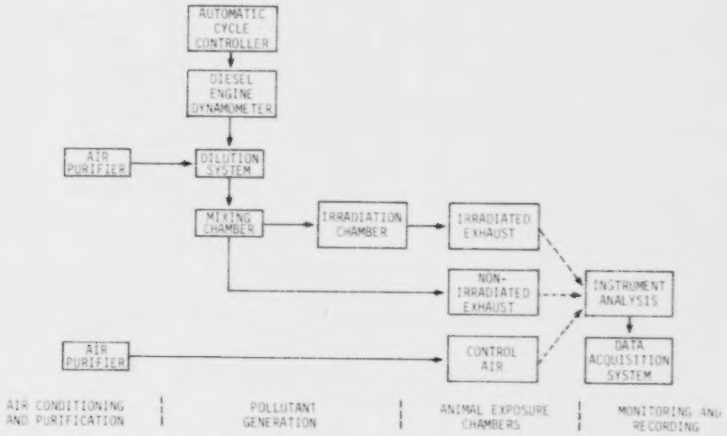

Figure 1. Schematic Flow Diagram
Diesel Exhaust System

Air Purifier. An air purifier unit provides, at maximum, 21.2 m^3/min. (750 CFM) of CBR (Chemical, Biological, Radiological) filtered and conditioned air to maintain the required temperature and relative humidity in the animal exposure chambers. Two units are required for dilution air for the diesel engine exhaust, and one for the clean air (control) animal exposure chambers. An air purifier system consists of a pressure blower, cooling coils, reheat coils, humidifier, and CBR filters combined into a single unit, with a separate refrigeration compressor and outside air cooled condenser. The sensors for the diluted raw exhaust are located downstream of the dilution tubes in order to correct for temperature and relative humidity changes when the hot and moist engine exhaust enters the system. The filtering system provides a pass through absolute filters to remove particles down to 0.3 micron (99.97% efficient) and pass through activated charcoal of a 20.3 cm (8 in.) bed depth. Exposure chambers on control air for both studies are supplied from the same CBR filtered source, with controls set to maintain 24°C (75°F) and 50% relative humidity in the animal chambers. The same air is also ducted to the air filter inlet of the engine being used, since a change in humidity affects the Nox emissions. There are a total of five (5) air purifiers with two pairs interconnected to provide 1500 cfm of dilution air for each engine study.

Engine. The diesel engine selected for the light duty mobile emissions study is made in Japan by the Nissan Motor Company (makers of Datsun automobiles). For long-term animal exposure studies, it is necessary to have automatic cycle control, and this in turn requires an automatic transmission. Mounted on a dynamometer base plate are two Nissan CN6-33 diesel engines coupled to Chrysler Torque-flite automatic transmissions Model A-727. The detailed engine specifications which incorporate the latest low smoke combustion head are as follows:

1. Four cycle, water cooled, naturally aspirated
2. Six cylinders in-line
3. Bore and stroke 3.27" x 3.94" (8.3 mm x 10.01 mm)
4. 198 cubic in. displacement (3244 cc's)
5. 92 maximum brake horsepower @ 4000 rpm.
6. Torque, maximum @ 2000 rpm-137.5 lb - ft
7. Compression ratio - 22 to 1
8. Approximate weight - 662 lbs.

The diesel engine used in the coal dust study is a Caterpillar model 3304 with twin disc clutch and Wagner water scrubber. This engine is very popular in mining work and some specifications are:

1. Four cylinders
2. Bore and stroke 4 3/4" x 6"
3. 425 cubic in. displacement
4. 100 horsepower (or 50 hp. derated)
5. Compression ratio - 17 1/2 to 1
6. Weight dry - 1640 lbs.

Dynamometer. One dynamometer base plate is used for the automotive diesel exhaust studies and another similar installation nearby is used for the coal mine diesel exhaust study. An Eaton-Dynamatic Model 758-DG dynamometer has been coupled to the transmission of the Nissan engine. The unit is an air gap, eddy current, dual rotational type that employs water cooling and has a power absorption rating of 50 horsepower in a speed range of 1,600 to 10,000 rpm. A 450-lb flywheel is directly coupled to the dynamometer to provide the rotational energy to simulate the inertial mass (load) of a moving 4000-lb vehicle. The dynamometer field current can be set at a constant value or made dependent on the flywheel speed. The dynamometer unit is located between the transmissions of the engines which can be coupled, one at a time, to the double-ended dynamometer. This feature provides insurance in case of mechanical breakdown during a long time chronic study, since another engine can be put on line in about 30 minutes to provide exhaust gas to the animal chambers.

Dilution Tube. The effluent from the engine exhaust system is passed through a muffler and flexible stainless steel (SS) pipe into the dilution tube. The 40.64 cm (16 in.) I.D. dilution tube is made from 16 gauge SS plate and conditioned air enters the tube from a remote supply source. At this point is a mixing baffle plate, with a 20.32 cm (8 in.) diameter hole bored in the center. The incoming dilution air, under pressure, is forced through this hole to mix with the raw exhaust. The tailpipe enters the dilution tube at a right angle to its axis and is elbowed 90^0 to become concentric with the dilution tube. The exit end of the exhaust elbow is in the same plane as the baffle. Located on the bottom and outside the dilution tube, at the baffle plate, are two quick-disconnect couplings. One allows the end of the flexible exhaust pipe from the muffler to connect with the dilution tube and the other connects to the outside atmosphere. A blank plug is installed in the disconnect to the dilution tube when the exhaust is vented outdoors, which allows interruption of animal exposures and provides opportunity to run the engine for test purposes without contributing exhaust emissions to the animal chambers.

By operating a damper in the air supply line, the dilution ratio can be controlled up to a maximum of 30 to 1. To retain the particulate matter in suspension and prevent condensation, it is necessary to dilute the whole exhaust with no less than 8 parts of air to 1 part of exhaust when cycling. The main portion of the dilution tube consists of a 4.88 m (16 ft.) long flanged section before continuing on through a duct of the same diameter and entering the mixing chamber. There are two dilution tubes and each is located overhead of the engine-dynamometer base plates.

Mixing chamber. The mixing chamber serves to attenuate concentration peaks in the nonirradiated exposure chambers and to avoid the problems associated with stream splitting and proportional sampling.

The diluted diesel exhaust enters the 19.34 m^3 (683 $ft.^3$) mixing chamber through a 40.64 cm (16 in.) diameter SS duct in the side wall. An elbow discharges the exhaust across the front of a tube-axial fan controlled at a low rpm by a Zero Max. unit, to mix the entering exhaust with the chamber atmosphere.

Pipes from the mixing chamber supply the exhaust either direct to the animal exposure chambers or to irradiation chambers enroute to exposure chambers. An adjustable pressure sensor controls a motorized damper in a vent line to the external atmosphere in order to maintain a positive pressure in the chamber. There are two identical mixing chambers located side by side on the mezzanine over the middle exposure chamber room.

Irradiation Chambers. The photochemical reactions that result from exposure of diluted raw exhaust to artificial sunlight take place in eight irradiation chambers. Fluorescent lighting panels composed of blue lamps, black lamps, and sun lamps outside the chamber pass intense ultraviolet radiation through windows of clear teflon FEP fluorocarbon film. Normal flow through the irradiation chambers is 0.71 m^3/min. (25 cfm) and requires approximately 3 1/2 hours to reach equilibrium at a constant inlet concentration. Each irradiation chamber has a column of 19.34 m^3 (683 $ft.^3$) and they are stacked in pairs to conserve floor space. An irradiation chamber has four banks of 66 fluorescent tubes each, mounted in four external cavity reflectors.

Exposure Chambers. There are 36 individual animal inhalation exposure chambers of two different configurations utilized in the diesel exhaust studies(2). Briefly, they are constructed on 14-gauge type 304 stainless steel and are cubical with formed funnels at the top and bottom of each chamber.
Twenty chambers of the 1.37 m (4.5 ft.) square size are divided into two groups, with twelve assigned to the mine diesel-coal dust project and eight to the automotive diesel exhaust study. Also, there are sixteen chamber 1.53 m (5 ft.) square size used only on the automotive exhaust study.

Ports are provided with PVC plugs that can be either removed or drilled for different uses that make access to the chamber necessary. Beneath the floor grating is a spray ring with eight wide-angle nozzles to flush the bottom funnel with warm water. A quick-opening lever operated gate valve at the bottom of the funnel is connected to a drain line for washing down the chamber.

The exhaust and air mixture is admitted tangentially to the short cylindrical section at the top of the chamber. As a result of a mild swirling motion, the air and test material are mixed and distributed uniformly throughout the chamber. The exhaust air is drawn axially from near the bottom of the chamber through an individual exhaust duct connected to the exhaust system.

A venturi calibrated for 15 air changes per hour is mounted between the flanges of the inlet tube and a butterfly damper. The pressure taps on the venturi are connected to a magnehelic pressure gauge calibrated for flow in cfm. The negative static pressure is measured with a magnehelic gauge connected to one of the port openings and regulated with a globe valve in the exhaust.

Exposure Chamber Rooms. There are four animal exposure chamber rooms with each of three rooms containing eight chambers used in the diesel auto exhaust studies and one larger room with twelve chambers for the exhaust and coal dust project. Each chamber room is individually air conditioned by a 5-ton self-contained air-cooled unit located outdoors. It is necessary to maintain controlled temperature in the exposure rooms in order to prevent heat transfer through the walls of the chambers and to avoid stressing the animals when they are removed from a chamber for examination or chamber cleaning. The floors are sloped to a trench drain at one end of the room and have a Dexotex covering to facilitate cleaning with a water hose. Each room is provided with a 24-inch wall exhaust fan for ventilation when chamber doors are opened and room lighting is on a timer for automatic day-night light cycle.

Engine Cycle. The dynamometer driving schedule for the Nissan engine consists of a repetitive series of idle, acceleration, cruise, and deceleration modes of fixed time sequences and rates. Table 1 shows this repetitive series of nine driving modes known as the Federal Short Cycle and used in the present fuel emission studies.

Fuel Selection and Storage. There are numerous federal military and commercial diesel fuel specifications and classifications used by industry, but those fuels marketed to meet the needs of heavy grade diesel fuel specifications are most commonly used. For automobiles, highway trucks, tractors, marine, and off road equipment, No. 2 Diesel is the typical fuel requirement and therefore was selected. The fuel was analyzed to contain 67.4% saturates, 3.4% olefins and 29.2% aromatics with 0.15% sulfur by weight. A fair comparison between the statutory emission levels and those of the engine under test should be made with a fuel that is representative of that in actual commercial use.

Two underground fuel storage tanks of 2250 gallon capacity are each equipped with electric fuel pumps to deliver fuel from the main storage supply to 10 gallon companion tanks located inside the building near the engine area. These tanks are mounted on platform weight scales to determine fuel consump-

Table 1

Federal Short Cycle

Mode	Time in mode (Secs)
0-16 mph acceleration	6
16-29 mph acceleration	23
29 mph cruise	10
29-37 mph acceleration	18
37-42 mph acceleration	4.5
42-37 mph deceleration	2.5
37-20 mph deceleration	32
20-0 mph deceleration	7.5
Idle	21.5
	125.0 seconds total

Table 2

Caterpillar Engine Cycle with 8 Steps

Mode #	Duration In Seconds	Speed %	Load %	Throttle %		Condition
1	20	82	50	72	@	2250 RPM 100% Load
2	20	98	75	70	@	2250 RPM 75% Load
3	40	98	50	65	@	2250 RPM 50% Load
4	40	98	25	65	@	2250 RPM 25% Load
5	20	98	0	63	@	2250 RPM 0% Load
6	80	98	98	57	@	2025 RPM 100% Load
7	20	98	98	47	@	1800 RPM 100% Load
8	360	98	0	0	@	IDLE 650 RPM 0% Load

600 Seconds Total

tion on a daily basis during the studies.

Coal Dust Aerosol. Generation and dispersion of the bituminous rank coal dust aerosol at a 2 mg/m^3 respirable dust concentration is accomplished with the Wright-dust-feed mechanism. Freshly micronized coal, reduced to a particle size 50% < 1 um, 90% < 3 um, is blown by the feed mechanism directly into the cylindrical section at the top of the exposure chamber, downstream from the diesel exhaust mixing chamber. The diluted exhaust-coal dust-air mixture, or coal dust-air mixture is dispersed uniformly throughout the chamber and drawn down past the animals' breathing zone.

INSTRUMENTATION

On Line Data Acquisition System for Monitoring Concentration of Gases. As shown in the block diagram (Figure 2), the atmospheric content from each chamber is sampled sequentially into a common manifold via On-Off solenoids activated by a digital programmable circuitry and controlled by a Hewlett-Packard 9830 calculator. The manifold is directly connected to On-Line analyzers for measuring carbon dioxide (CO_2), carbon monoxide (CO), total hydrocarbons (THC), sulfur oxides (SO_x), nitric oxides (NO), nitrogen oxides (NO_x). The operation of sampling the analyzers is controlled by the programmed calculator. The solenoid of a chamber is activated, and the atmosphere of that chamber enters the manifold. At this point in time there is a 3 minute period allowed for the atmosphere and the analyzers to stabilize. At the end of that time, the outputs of each analyzer are fed sequentially to a Digital Voltmeter. The data in digital form is transferred to the memory of the calculator where it is stored. When the data from the last analyzer has been stored in the memory, the calculator commands the digital circuitry to switch-On the next solenoid which opens the atmosphere of the next chamber to the manifold, and the sequence is repeated.

There are 16 chambers in the automotive engine study to be monitored and scanning of all the chambers takes 1 hour. Therefore, for an 8 hour day there are 8 scans per day i.e., 8 measured data points for each gas in each chamber. The statistical analysis of the data can be done at any time for any period of time for any chamber or gas. The analyses program converts the data entered into memory in volts into values in parts per million. The printout of analysis contains the following information: date, chamber number, name of the gas, treatment of the atmosphere, number of data points monitored, average value in parts/million, maximum value, minimum value, standard deviation, standard error, and confidence limits. In general the printout of measured and analyzed data is reported on a weekly basis.

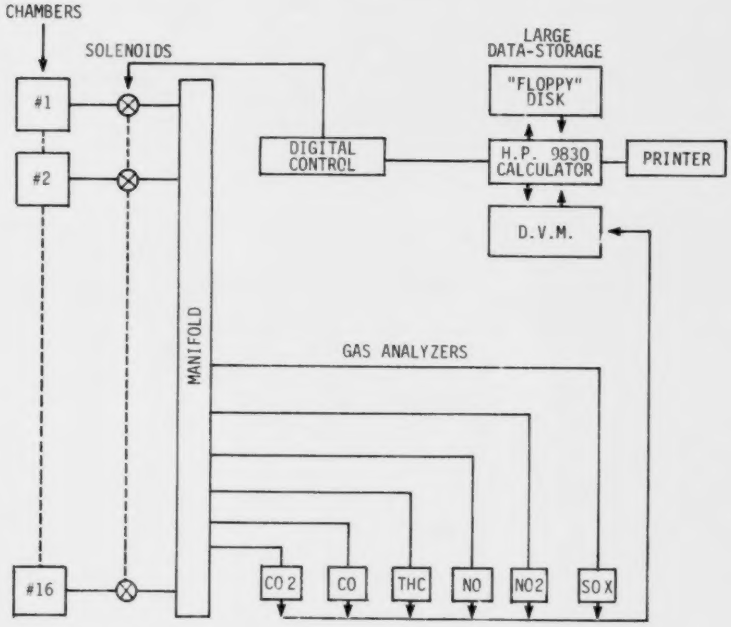

Figure 2. Block Diagram Data Acquisition System

EXPOSURE CHAMBER AEROMETRY

Characterization of the pollutant atmospheres helps relate the animals exposure effects to the particular agents present in those atmospheres. On a more general basis, the differences between the irradiated and nonirradiated atmospheres are determined and involve both the gaseous and particulate phases of each atmosphere. The analyses provide for measurements of ammonia gas and product ammonium compound resulting from chamber animal contributions interacting with the other atmospheric components.

Table 3, on analytical measurements, lists the method or principle and the specific instrument used for each of the components considered. The principal, regulated (i.e., pub-

Table 3

Analytical Measurements of Exposure

Chamber Atmosphere Components

Component		Principle	Instrument
Carbon Monoxide	CO	NDIR	Beckman 865
Carbon Dioxide	CO_2	NDIR	Beckman 865
Nitrogen Oxides	NOx	Chemiluminescence	TECo 14T
Nitric Oxide	NO		
Nitrogen Dioxide	NO_2		
Total Hydrocarbons	THC		
Ambient Temp		FID	Beckman 400
High Temp. (350° F)		FID	Beckman 402
Hydrocarbons/GC		Gas Chromatography	Beckman 6800
Methane	CH_4		
Non-Meth HC	NMHC		
Ethane	C_2H_6		
Ethylene	C_2H_4		
Sulfur Dioxide	SO_2	Pulsed UV Fluorescence	TECo 43
Ozone	O_3	Chemiluminescence	Meloy 320
Ammonia	NH_3	Phenol-Hypochlorite	Beckman DB
Aldehydes	CHO's	DNPH/GC	Perkin-Elmer 900
Particulate, Mass	M	GF Filter/Gravimetric	
Sulfate	$SO_4^=$	Ion Chromatography	Dionex 14
Ammonium	NH_4	Ion Chromatography	Dionex 14
Nitrate	NO_3^-	Ion Chromatography	Dionex 14
Chloride	Cl	Ion Chromatography	Dionex 14
Particulate Size	MD	Scanning EM	
Polynuclear Aromatics	PNA's	Liquid Chromatography	
Coal Dust	Total & Respirable Mass	Filter/Gravimetric	

lished in the Federal Register) components - CO, NO_x and THC - as well as CO_2 and even sulfur dioxide (SO_2) when necessary, are monitored continuously. A system of teflon tubing lines leading from the chambers to a group of solenoid valves supplies a sample of each chamber atmosphere in turn to the several monitoring instruments. Other atmospheric components are sampled and measured at various intervals.

Method of Measurement.

A. Gaseous Components

1. The CO, CO_2, NO and NO_2 measurements are made at regular intervals during the day for each of the exposure chambers with the exhaust emissions atmosphere. The interval between successive samples from one chamber and the next was determined by the rise time in responses collectively considered for the various instruments. Filter holders equipped with glass fiber filters are installed at the probe ends of the teflon supply lines, i.e., at the exposure chambers, in order to remove particulates from a gas sample as a means of protecting the supply lines and the instruments against deposition and contamination by such particulate.

2. In addition to THC values obtained at the room temperature of the instrument room by continuous monitoring, there is provision for daily measurements of heated-probe THC from selected exposure chambers. The flame ionization detector (FID) instrument is fitted with a short-heated (350° F) sampling line in the immediate vicinity of the chamber to minimize line (adsorption) losses.

3. Individual hydrocarbons (methane, nonmethane hydrocarbons as a group, ethane and ethylene) are monitored at irregular intervals by the gas chromatograph (GC) in the instrument room. Gas sample supply lines, separate from the solenoid-controlled system for the regulated gases mentioned previously, are used to feed the gases continuously from the exposure chamber to the gas chromatograph. These individual organic components may serve as markers for the saturated and the unsaturated organic class groups.

4. The pulsed UV fluorescence (PF) instrument is convenient for monitoring SO_2. However, because of projected interference from other diesel engine exhaust emissions components, an adsorption device is used to eliminate such interferents and the instrument is used on a discontinuous basis to prolong the life of the adsorptive agent. The West Gaeke(3)

colorimetric method applied to bubbler samples serves as a back-up procedure in case of the arrest of the PF instrument.

5. Ozone (O_3) measurements are taken at the large irradiation chambers, with occasional checks on the exposure chamber atmospheres when applicable.

6. For determination of ammonia gas (NH_3) concentrations in the more heavily animal populated exposure chambers, bubbler samples may be collected for analysis by the phenol-hypochlorite method according to Weatherburn(4). A chemiluminescence (NO_x) instrument provides an alerting system for high (above 1 ppm) peaks of ammonia concentrations.

7. The dinitrophenyl hydrazine (DNPH) method(5) for individual aldehydes is used in place of the methylbenzothiazolone hydrazone (MBTH) method(6) for aldehydes as a group; the latter has been considered to be inadequate in diesel emissions measurements. Bubbler solutions of the DNPH collecting reagent are immersed in ice baths during the samplings to improve collection efficiency. The bubbler samples are subsequently analyzed by a GC procedure for specific aldehydes, namely, formaldehyde, acetaldehyde, the propionaldehyde-acetone group, crotonaldehyde and hexanaldehyde.

B. Particulate Components

Engine Organic Compounds.

1. Particulate mass measurements are made daily from filter samplings obtained from the exposure chambers with the exhaust emissions atmospheres, as well as from the dilution tube.

2. Aqueous extracts of the particulate filter samples are analyzed by ion chromatography for various cations and anions, namely, the ammonium and the sulfate ion concentrations.

3. Hi-vol samplings are made occasionally to provide large enough samples for analysis of the organic make-up according to functional groups, Figure 3.

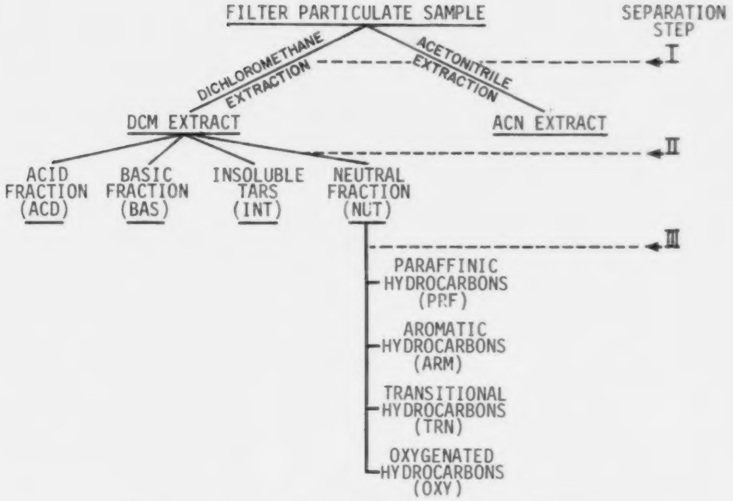
FILTER PARTICULATE SAMPLE
SEPARATION STEP
DICHLOROMETHANE EXTRACTION
ACETONITRILE EXTRACTION
I
DCM EXTRACT
ACN EXTRACT
II
ACID FRACTION (ACD)
BASIC FRACTION (BAS)
INSOLUBLE TARS (INT)
NEUTRAL FRACTION (NUT)
III
PARAFFINIC HYDROCARBONS (PRF)
AROMATIC HYDROCARBONS (ARM)
TRANSITIONAL HYDROCARBONS (TRN)
OXYGENATED HYDROCARBONS (OXY)

Figure 3. Separation Scheme for Diesel Engine Particulate Organic Compounds

The filters are extracted by methylene chloride (DCM) and then by acetonitrile (ACM), step I. The extracted organics are treated in a separation scheme, step II, to produce the acidic (ACD), basic (BAS) and neutral (NUT) fractions as well as an insoluble tar (INT) portion. With the major interest in the neutral fraction for mutagenic and carcinogenic potential, that fraction is further treated in gravity flow liquid chromatography, step III, to yield the paraffinic (PRF), aromatic (ARM) and oxygenated (OXY) hydrocarbons groups as well as a color-band visible transitional (TRN) hydrocarbons group. Additional procedures such as high pressure liquid chromatography (HPLC) can be used to resolve individual components in the various fractions as well as class groups in the mother extract (DCM, ACN).

4. Particle sizing of the particulate is demonstrated in samples collected on Nucleopore filters which lend themselves to being treated for convenient viewing by a scanning electron microscope.

The Electrical Aerosol Size Analyzer (Thermo-Systems Inc.) is adaptable to particle size distribution measurement but is used minimally because of the very "dirty" nature of the diesel emissions which can readily foul up the instrument flow component parts.

5. Coal Dust. Chemical and physical characterization is made of the coal dust before and during the duration of the study. Chemically, inorganic and organic content are determined and include vanadium, arsenic, lead, cadmium, iron, nickel, aluminum, zinc, beryllium, chromium, copper, manganese, cobalt, free and combined silicon dioxide. Particle-size distributions are determined as well as adsorptive characteristics between coal dust and diesel exhaust.

Respirable particulate and total mass measurements are made daily from periodic samplings obtained from the exposure chambers. Collections are made on Gelman Metricel VM1 filters, 5.0 um pore size, and analyzed gravimetrically to determine dust concentration.

C. Analytical Measurement Values
The data from the various analyses is used to supply whole study averages and also periodic averages for the exposure chamber atmospheric pollutant concentrations.

The whole study averages give, for example, a single CO concentration value for all of the CO measurements made on all of the irradiated atmosphere exposure chambers.

The same data bank that supplies the whole study averages if segmented into weekly averages that can readily be plotted as a bar graph, for instance, to show the trend of component concentration levels over the lifetime of the study. Such a trend plot may conveniently show the rate of change as well as the total change in composition as a result of changes, for example, in engine combustion efficiency, in fuel supply (composition), or even in animal loading in the exposure chamber(s). The trend also may confirm, by its consistency in differences between the irradiated and nonirradiated atmosphere concentrations, a real, though subtle difference in the whole study averages for the two types of atmospheres.

HEALTH EFFECTS

The primary health concern regarding the use of diesel engines involves the particulate fraction of diesel exhaust. The nature of the diesel particulate is potentially of more significance than just the total mass of particulate emissions, which are up to 82 times as much as in gasoline exhaust using paired vehicles(7). The particulate is principally small agglomerated chains of very small carbon spheres which are frequently coated with adsorbed condensed material mostly in the form of high molecular weight organic compounds, sulfur dioxide, sulfates and materials such as polycyclic aromatic hydrocarbon (PAH)(8). Particulates contribute to health effects by carrying the irritants to the deeper, more sensitive recesses of the lung where they can act to initiate, promote, or accelerate emphysematous, fibrotic, or carcinogenic processes(9). There are several important reasons why increased exposure to the particulates in diesel exhaust may constitute a potential health hazard(10): a) the carbonaceous particle is basically a high molecular weight, polycyclic aromatic hydrocarbon; b) the particles have a high surface area, theoretically enabling them to adsorb large quantities of exhaust gases, most importantly the carcinogenic polycyclic organic matter (POM) such as benzo(a)pyrene; c) its low settling velocity in air allows time for atmospheric oxidation and degradation to lower molecular weight POM's which are potentially carcinogenic; d) the particles are primarily in a size range (0.2 - 0.3 um mean diameter) which would allow deep penetration into the lung and may retard the lung's clearance defense mechanisms once deposited.

The use of diesel engines in underground coal mines is being encouraged in order to escape the tragedy of fire and explosions resulting from arcing of presently used electric motors and cables. Organized mine labor is opposed to the use of diesels until biological effects data are available from animal studies using diesel exhaust after it has passed through a scrubber.

To determine if there are potential health hazards arising from the operation of diesel engines in coal mines, this project has four specific objectives. The first is to determine if combined exposure to diesel exhaust and coal dust changes the incidence of neoplastic disease, as compared with controls and to exposure of each alone. The other three objectives are investigation of pulmonary response, immune potential and mutagenic potential of combined exposure to coal dust, diesel exhaust and each agent alone in comparison to control animals.

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BEHAVIORAL ALTERATIONS DUE TO DIESEL EXHAUST EXPOSURE

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ABSTRACT

Several experiments examining the effects of diesel exhaust on the behavior of rats are reported. Animals were exposed either as adults or neonates. The spontaneous locomotor activity (SLA), measured in standard running wheel cages, of adult rats exposed for 8 hours per day, 7 days per week was significantly less than that of controls. Experiments involving diesel exhaust exposure to neonatal rats indicated that adult rats, exposed to diesel exhaust during their neonatal lives, were significantly less active as measured by SLA. Adult rats, exposed to 20 hours diesel per day as neonates, were placed in skinner boxes after the SLA experiment described above had been completed. The exhaust exposed animals showed significantly decreased acquisition of a food reinforced bar pressing task. All animals that learned this task extinguished at the same rate. The results of the neonatal diesel exhaust experiments support the hypothesis that diesel exhaust exposure during development of an organism can lead to behavioral differences in adulthood.

INTRODUCTION

These experiments were conducted as part of of an overall EPA program studying the health effects of diesel exhaust. The objective of this section of the program was to measure the behavioral consequences of diesel exhaust exposure to adult and neonatal animals. Behavior was used as a measureable end point because it can be general indicator of the health status of an animal.

It was previously reported(1) that exposure of rats to approximately 6 mg/M^3 diesel exhaust for 20 hours per day, seven days per week significantly reduced two types of spontaneous activity: 1) adult spontaneous locomotor activity, and 2) neonatal pivoting. The work described in this paper is a continuation of the study of the effects of diesel exhaust exposure on behavior.

Two sets of experiments are reported in this paper. The first set examines the hypothesis that the depression in running wheel behavior previously observed in adult rats exposed to diesel exhaust for 20 hours per day would be less severe in adults exposed to only eight hours per day. The second set of experiments examine the hypothesis that neonatal exposure to diesel exhaust will produce alterations in adult behavior. In conjunction with one of the neonatal experiments, electrophysiological recordings were collected from litter mates of animals used in the behavioral experiments. The results of the electrophysiological experiments are presented in a companion paper(2).

METHODS

EXPERIMENT INVOLVING EXPOSURE OF ADULT RATS

Exposure. The diesel exhaust group was exposed for eight hours per day (6:30 am to 2:30 pm), seven days per week, to diesel exhaust generated by a Nissan CN6-33 engine coupled to a Chrysler Torque-flite automatic transmission, Model A-727. The raw exhaust was diluted with filtered air in a ratio of approximately 1:20 to achieve a particulate matter concentration of 5.97 ± 0.17 mg/m^3 during the 16 weeks of exposure (weeks 10 through 25 of the EPA program reported by Hinners, et al. (3). Concentrations of the measured components of the diesel exhaust are listed in Table 1. The control group was exposed to filtered air only.

Table 1

Average Levels (± S.E.M.) of Diesel Exhaust Components During Adult Exposures

COMPONENT	CONTROL AIR	DIESEL EXHAUST
CO	1.86 ± 0.06	19.20 ± 0.35
CO_2(%)	0.05 ± 0.00	0.28 ± 0.01
Hydrocarbons	3.22 ± 0.08	7.29 ± 0.11
NO	0.08 ± 0.01	11.14 ± 0.43
NO_2	0.03 ± 0.00	2.51 ± 0.10
Particulate (mg/M^3)	0.01	5.97 ± 0.17
SO_2	0.46 ± 0.02	1.82 ± 0.07

Spontaneous Locomotor Activity. Spontaneous locomotor activity (SLA) measurements were obtained by placing adult Charles River Sprague Dawley rats (45 days post parturition) into standard, 14 cm running wheels. Two weeks of baseline data were obtained for 50 rats, housed in a controlled environment. In an attempt to reduce the amount of variability in the data, the very high and very low runners were excluded. Of the original 50, 26 rats were pair matched for SLA levels, the group size being limited by the size of available exposure chambers. One rat from each pair was randomly assigned to either the control air or diesel exhaust group. The remaining rat from each pairing was placed in the opposite exposure situation.

The rats remained in their respective chambers for 16 weeks. Rats received standard laboratory chow and tap water ad libitum. Each week body weights were taken and the food and water weighed and changed. The chambers were hosed daily and scrubbed with "Scum Remover" weekly. The reading from the mechanical wheel revolution counter was recorded daily.

EXPERIMENTS INVOLVING EXPOSURE OF NEONATAL RATS

Exposure. There were three neonatal exposures to diesel exhaust. Table 2 lists the three exposures and indicates the length of exposure per day, the age of the neonates during their exposure, the number of litters and the number of animals per group. The concentrations of the various exhaust components were similar to those during the adult exposure experiments.

Table 2

Exposure Conditions for Neonatal Rat Experiments

Exposure Situation	Hrs. of Exposure per day	Age of Neonates During Exposure (Days post paturition)	# of Litters per Group	# of Animals per Group
1	20	1-17	5	12
2	8	1-21	12	15
3	8	1-28 or 42	14	28

Animal Care. For each of the neonatal experiments, timed pregnant Sprague Dawley rats were ordered from the animal supplier. The day after parturition each litter was culled to eight pups containing either four, five or six males. The litters were then pair matched for dam weight, litter size, male to female ratio, pup weight, length of gestation and time of day parturition began. One member of each pair was randomly assigned to either the control air or diesel exhaust exposure condition and the remaining litter received the other treatment. After the exposure schedules depicted in Table 1 were concluded, the litters were removed from the chambers, transferred to another building and reared in a standard laboratory enviornment.

Spontaneous Locomotor Activity. At the age of six or seven weeks post parturition, the neonatally exposed animals were placed into standard running wheels and measurement of SLA began. Readings from the wheel revolution counters were recorded daily. Body weights and food and water consumption were monitored weekly.

Bar Pressing Task. When the rats exposed to 20 hours diesel exhaust per day as neonates (Exposure situaiton 1 of Table 1) were 15 months of age, an attempt was made to train them to press a bar for food pellet reward. Prior to the onset of training the rats were placed on a food restricted diet until they achieved 80% of their previous body weight. Upon reaching the 80% criterion the animals were placed in an enclosed, ventiliated 30 x 30 cm Skinner Box containing a bar press mechanism adjacent to a food pellet cup on one wall of the chamber. Standard psychological shaping procedures were used to train the rats to bar press. Following acquisition of of the task, performance was maintained on a continuous reinforcement schedule (CRF). After 42 days of CRF, the task was switched to an extinction condition in which the rats were placed in the boxes but did not receive food pellets for bar presses.

RESULTS

ADULT RAT EXPOSURES

The SLA data obtained from rats exposed to diesel exhaust as adults are represented in Figures 1 and 2. Both groups show a typical inverted U shaped pattern of running wheel behavior over time. During the 2 week baseline period (Figure 1), the control and experimental groups are similar because of pair matching. Week 3 SLA was much lower than week 2, probably as a result of the movement of the animals into the exposure chambers. As the experiment progressed, both groups showed increased SLA, with the control group tending to be more active than the pair matched exposed group. However, the only period in which the group differences reached statistical significance was week 11 of the experiment. The data were also analyzed in an alternate method by expressing each animals SLA as a percentage of the two week baseline period. In this analysis, each animal serves as his own control, and the variability of the data is reduced. As depicted in Figure 2, when SLA is expressed as a percentage of baseline, the diesel group is significantly less active than the control group during 4 weeks of the experiment (weeks 8, 9, 11 and 12). Significant decreases in food and water consumption for the exposed group correlated with decreased activity trends. Body weights were not significantly different.

NEONATAL RAT EXPOSURES

SLA of Rats Exposed for 20 Hours Per Day. Figure 3 consists of the adult SLA data from rats exposed to diesel exhaust for 20 hrs/day as neonates. Both groups show a typical pattern of running wheel behavior over time, however the exhaust exposed animals were significantly less active than control animals from week 5 to the end of the study.

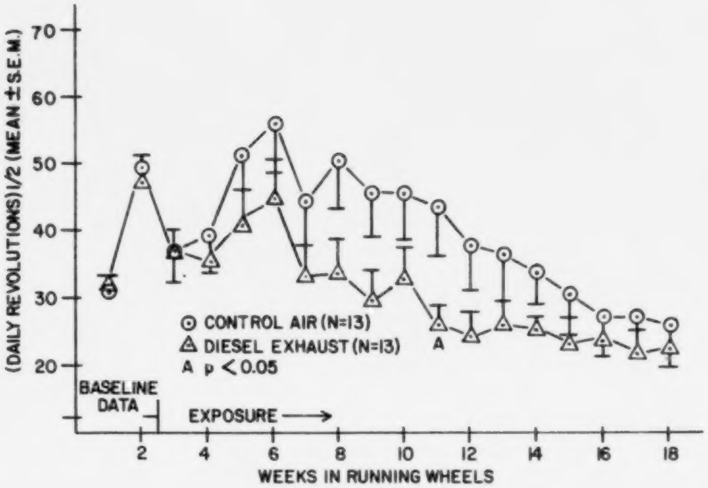

Figure 1. Spontaneous locomotor activitiy (SLA) of rats exposed to either control air or diesel exhaust (8 hours per day (0630 to 1430) SLA measurements began 6 weeks post parturition (pp). Exposure began 8 weeks pp.

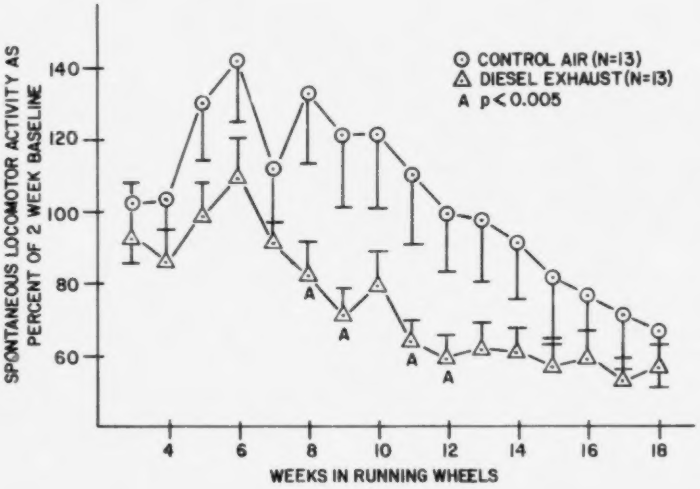

Figure 2. Spontaneous locomotor activity (SLA) (expressed as percent of 2 weeks baseline) of rats exposed to either control air or diesel exhaust (8 hours per day (0630 to 1430). SLA measurements began 6 weeks post parturition (pp). Exposure began 8 weeks pp.

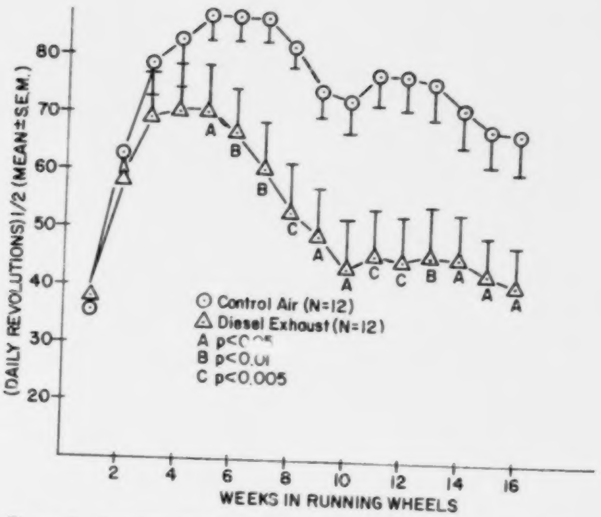

Figure 3. Spontaneous locomotor activity (SLA) of rats exposed to either control air or diesel exhaust (20 hours per day, days 2 through 18 post parturition (pp). SLA measurements began 7 weeks pp.

SLA of Rats Exposed for 8 Hours Per Day. The SLA data from the first 8 hrs/day neonatal exposure experiment (Table 2, Exposure Situation 2) was highly abnormal in character. Neither control nor exposed animals demonstrated a typical inverted U shaped function over time. Activity levels for both groups remained at a low level throughout the experiment, with control animals running only 17% as much as the controls in the previous experiment (Figure 3). The SLA values for control and exposed animals for this experiment were not significantly different. An adequate explanation for the low SLA values demonstrated by both groups in the first 8 hr/day experiment has not been reached.

A second experiment exposing neonates to 8 hours diesel exhaust per day was conducted because of the abnormal data from the first 8 hr/day experiment. The second experiment contained 2 diesel groups, exposed from days 1 to 28 or 1 to 45 post parturition, and 2 corresponding control groups (Table 2, Exposure Situation 3). There were no significant differences within either exposure condition. Therefore, the data from the two exposure and the two control groups were pooled to achieve the groups represented in Figure 4. Both control and exposed groups showed a typical inverted U shaped SLA function over time, with the control group demonstrating a normal level of SLA. The neonatally exposed group was significantly less active than controls during each week of the experiment from weeks 5 to 13, with the exception of weeks 7 and 11.

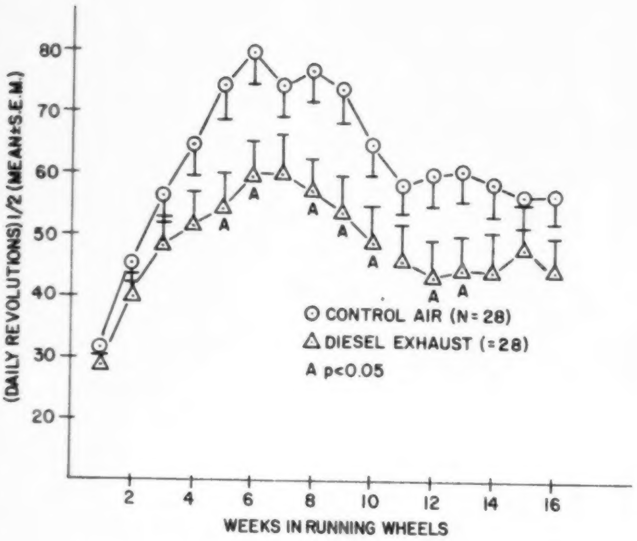

Figure 4. Spontaneous locomotor activity (SLA) of rats exposed to either control air or diesel exhaust (8 hours per day, days 1 through 28 or 45 post parturition (pp). SLA measurements began 6 weeks pp.

Bar Pressing Acquisition for Rats Exposed for 20 Hours Per Day. The results of the bar pressing acquisition task are presented in Figure 5. After a few experimental sessions, animals in the control group began to press the bar. With continued experience in the task, the control animals showed a steady increase in the number of bar presses registered per day. In contrast, the diesel exposed group acquired the task only after extensive training. After 25 days of shaping, only one of the 10 diesel animals had learned to press the bar. With continued training most of the diesel animals did begin to bar press. However, after 42 days of shaping 2 of the 10 had not acquired the task. From the 13th to the 42nd day of the experiment, the diesel group registered significantly fewer bar presses per day than the control group. When switched to extinction conditions on day 43, both groups showed a rapid decline in the number of bar presses registered per day. There were no statistically significant differences between exposed and control groups during extinction.

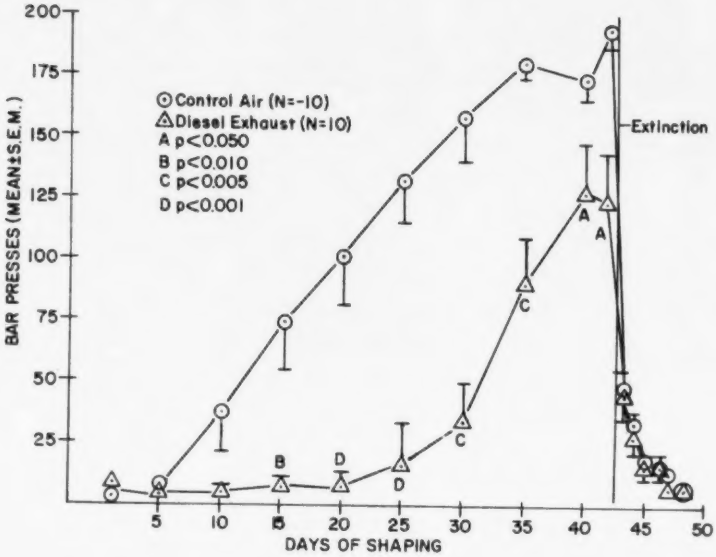

Figure 5. Bar presses for food reward of rats exposed to either control air or diesel exhaust (20 hours per day, days 2 to 18 post parturition). Training began at 15 months of age.

DISCUSSION AND CONCLUSIONS

ADULT EXPOSURE

Exposure to diluted diesel exhaust lowered the SLA of adult rats. In the previous study(1), rats exposed to 20 hrs/day diesel exhaust (8:00 pm to 4:00 pm the next day) failed to show a typical inverted U shaped running pattern over time, and were significantly less active than controls each week of the 6 week exposure period and one week afterward. In contrast, rats exposed to diesel exhaust 8 hrs/day did show a typical increase and subsequent decrease in SLA over time, and were significantly less active than controls for 1 or 4 weeks of the experiment, depending on the data analysis. The greater effect of the 20 hr/day exposure could be due to two factors. Not only were the 20 hr/day animals exposed to a greater amount of diesel exhaust per day, but the exposure occurred during the night time which is the period of highest activity for the rat. In contrast, the 8 hr/day exposure occurred during a period of the day during which rats are normally quiescent. It follows that the greater depression of SLA found in the 20 hrs/day group could be due to either a greater dosage of exhaust, or to exposure during the period of high SLA activity, or both. The fact that SLA depression was found in the 8 hr/day group who were not being exposed to exhaust during the period of the night when most SLA occurs suggests that the depression must have been caused by a residual effect of the exhaust. The residual effect could have been either due to some components of the exhaust remaining in the chamber after the exhaust was turned off, or due to some effect on the animals themselves.

A comparison can be made of adult SLA data obtained from diesel exhaust experiments with similar data obtained from automobile exhaust experiments(4). For example, the 20 hour per day exposure to an 18:1 dilution of diesel exhaust resulted in SLA that was only 7% of control level. Experiments involving 24 hours per day exposure to an 11:1 dilution of untreated or catalytically treated automobile exhaust reduced the exposed rat's SLA to only 60% of the control level. Although comparisons of this sort must be made with caution considering inter-experiment variability, these experiments provide evidence suggesting that diesel exhaust is more potent at reducing SLA than automobile exhaust.

Based on reports in the literature(1,4-7), several individual components of the exhaust, including CO, H_2SO_4, NO, NO_2, and SO_2 can be ruled out as causative factors in the reduction of SLA in exposed adults. None of these compounds suppresses SLA to an extent comparable to diesel exhaust. This leads to the conclusion that the components of the exhaust that are responsible for the observed effects are either the hydrocarbons, particulate matter or some combination of the single constituents. In order to discern whether the effects are due to the gaseous components or the particulate matter, it would be beneficial to examine the effects of exposure to whole exhaust and whole exhaust minus the particulate matter as compared to control air.

NEONATAL EXPOSURES

Two of the neonatal experiments (Table 2, Exposure Situations 1 and 3) suggest that there may be permanent sequalae associated with diesel exhaust exposure during the neonatal life of the rat. As adults, the two groups of animals exposed to diesel exhaust during development were significantly less active than their respective control groups. In addition, it can be seen that the differences between the control and exposed groups were greater for the 20 hour per day exposure versus the 8 hour per day exposure. This suggests a dose-response relationship that is qualitatively similar to the adult effect.

The results from Exposure Situation 2 of Table 2 are difficult to interpret because of the unusually low activity levels displayed by both control and exposure groups. Neither group demonstrated signs of illness or other possible reasons for the failure to acquire normal running wheel behavior patterns. Whatever the case may be, this particular experiment cannot be taken as positive or negative evidence of the health hazards of diesel exhaust exposure.

The difficulty the diesel exhaust animals (Exposure Situation 1 of Table 2) had in acquiring the bar pressing task is further evidence that exposure to diesel exhaust during development may produce long lasting differences in behavior. The nature of the behavioral deficit is not clear. It cannot be determined from this experiment whether the differences in task acquisition are due to a learning deficit or to some other reason (e.g. motivational or arousal differences).

At this time, it is impossible to attribute the effects of the neonatal exposure to any particular constituent(s) of diesel exhaust. There is little comparable research. Components such as CO, hydrocarbons and particulate matter may play an important role in causing the observed behavioral differences.

FUTURE RESEARCH

To date, risk assessment for diesel exhaust exposure has concentrated on the possible oncogenitic properties of the exhaust. The research reported here indicates that there are long lasting effects attributable to diesel exhaust exposure in addition to carcinogenicity. In order to fully assess the risk of exposure, all potential health problems must be investigated. Many projects could be proposed that would contribute to the understanding of the behavioral consequences of exposure to diesel exhaust. 1) Dose-response relationships should be examined in depth; 2) Various constituents of diesel exhaust should be examined to determine their relative contribution to the observed toxic effects; 3) Effort needs to be expended in development of human risk assessment models based on behavioral data; 4) The site of the lesion(s) should be localized; 5) Species differences need to be evaluated; and 6) Comparisons between diesel and gasoline engines need to be made, especially relative to the neonatal effects.

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General Discussion

J. VOSTAL: About a year and a half ago, we were together with our colleagues from EPA in a meeting in Houston where the first study about the potential effects of diesel exhaust on the behavior of animals were reported. I am a little surprised since I have heard practically the same type of conclusion today that I heard two years ago. You are saying that you cannot distinguish what type of component of diesel exhaust is responsible for the behavioral effects. This was the same thing that we heard at that time. You are saying that you don't know if the effect was caused by a direct effect on the central nervous system or indirect on another system. Could I ask what is the objective? Do you expect that the objective of this study to be the effects on rats living near the tailpipe of a diesel engine? Did you measure any levels of carboxy hemoglobin? Did you try to do any type of positive test using only carbon monoxide?

W. BOYES: Results that are new include the extent of the behavioral deficit in a learning situation. We have also monitored neurophysiological development of rats. We do know that there is a nervous system lesion involved. There are a number of studies planned that examine the different constituents of diesel exhaust. So far, most studies have only been done using adult animals, not neonates. Unfortunately, we haven't had the opportunity at this date to measure additional parameters.

D. LACHTMAN: Are there similar studies to the one you reported in the literature with respect to NO_2 and CO that have been reported?

W. BOYES: With regard to the adult exposures, there are a number of experiments with carbon monoxide and nitrogen oxide. The adult deficit in behavior does not appear to be due to those constituents of the exhaust. Nor does it appear to be due to the SO_x. The deficit is probably due to the particles or the hydrocarbons. With respect to neonatal exposure, there has not been work done that would compare to our results, and we do not know which constituents of the exhaust may be causing the effects.

D. LACHTMAN: Again, what was the NO_2 level?

W. BOYES: About two and a half parts per million.

J. HAFELE: That is a very interesting experiment. Have you thought of doing similar kinds of experiments with other types of engines, for example, gasoline engines?

W. BOYES: Yes, we would like to compare the diesel to the gasoline engine, but we don't have the facilities to do it at this moment.

J. HAFELE: One might be able to conclude that any type of engine could cause this kind of behavior, and it is not peculiar to diesel. I quarrel with the title of your conclusions.

W. BOYES: With the title that it is due to diesel exhaust?

J. HAFELE: Yes, that it is due to diesel exhaust. I would tink that there are components that are similar to other types of engines as well as could cause that kind of behavior.

W. BOYES: Similar studies have been done with adult animals exposed to catalytically and noncatalytically treated gasoline engine exhaust. It is hard to compare from one study to another. But, it is generally true that the effects we find in diesel animals are more marked than those found in adult rats exposed to gasoline exhaust. Gasoline exhaust does produce a depression in activity. Effects have not been studied in neonatally exposed animals.

J. HAFELE: Six milligrams of particulate probably is about a dilution of five or 10 to one.

W. BOYES: It is about 18 to one.

J. HAFELE: Similar dilutions for other exhausts would be preferred, I would think.

W. BOYES: We would like to examine the different constituents of the exhaust in neonatal animals and see if we can identify which constituents are producing the effects.

R. SCHRECK: During some of our earlier developmental work, I had occasion to be in chambers that had lower concentrations of diesel exhaust than this. In about 30 seconds my eyes began to water, I was crying, and I could hardly get the instruments set before I got back out again. I am wondering if you considered the fact that animals may have been so sensory withdrawn from that expsoure that they may not have sought stimuli during this still-developing period of their CNS. Therefore, they had not had very much of a sensory experience . Something like an acrolein exposure, tear gas, restraint, or some other type of an effect that may have nothing to do with diesel particles per se. These factors may give you the same kind of effect. I was wondering if you have done studies like that?

W. BOYES: No, we haven't. That is one way that the effects could be manifest, although I am sure there are other ways as well.

B. SOCAL: The shaping delay that you had was very interesting, but it might not be too conclusive. As a suggestion, I might recommend that you try a new learning task. You have gone through shaping and extinction and maybe you should try a second task as well.

W. BOYES: Yes, I would caution people not to conclude that this is a learning deficit. There could be many factors which influence the learning of a rat. For example, the motivation to learn could occur at 80 percent of their previous body weight. Perhaps the exposed animals weren't motivated to learn, because they weren't hungry enough. There are other factors as well. We do plan to study other types of learning tasks with different motivations; for example, shock motivation, maze learning etc. If we do those experiments, then we will be able to determine if there is a learning defect. Thus, at present, we have not yet sufficiently characterized the nature of the behavioral deficit.

RIGGER: I would like to follow-up on the gentleman's question relating to animals exposed to gasoline emissions since it has been shown that animals thus exposed may have behavioral changes, I might ask whether the remarkable disparities in results between exposed and nonexposed, are out of line with expectations?

W. BOYES: From which type of exhaust?

RIGGER: Related to comparably exposed rats from gasoline or other pollutants, would you expect results similar to what was found in these diesel exposed animals?

W. BOYES: With regard to the adult exposures?

RIGGER: Yes.

W. BOYES: They are in line with what we would expect in comparison with gasoline engines. As I said, gasoline emissions do produce a depression in running behavior. It is hard to compare the absolute magnitude of depression from one study to another. It appears that the diesel exhaust produces a greater reduction in adult running wheel behavior than gasoline exhaust. We don't have much literature to compare the neonatal exposure studies to, so it is hard to draw conclusions relative to expectations.

NEUROPHYSIOLOGICAL ALTERATIONS DUE TO DIESEL EXHAUST EXPOSURE DURING THE NEONATAL LIFE OF THE RAT

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ABSTRACT

This study was designed to assess the effects of diesel exhaust on the development of the nervous system in rats as measured by somatosensory and visual evoked potentials (SEPs and VEPs, respectively). SEPs, elicited by 1 mamp, 0.5 msec pulses delivered to the tibial nerve at the tibale, were recorded from the skull overlying the somatosensory projection area on days 28, 35, 42 and 49 postconception (pc). Analysis of the data for day 35 pc double pulse SEPs revealed significantly longer latencies for all peaks of the SEP in pups exposed to diesel exhaust. There were also differences indicating that the electrophysiological recoverability of the nervous system had been compromised in the diesel exhaust exposed group. On day 35 pc, the latencies of all VEP peaks occurring before 300 msec were greater in the exposed group. The differences, however, reached statistical significance only for the P2 peak of the response to the first of the paired stimuli. These data support the hypothesis that diesel exhaust influences the development of the nervous system.

INTRODUCTION

Data from a previous experiment revealed that exposure to diesel exhaust during the neonatal life of rats delayed the day of eye opening and reduced the amount of pivoting behavior displayed on days six and seven postparturition (1). Two further experiments, reported in a companion paper (2), indicated that the behavioral alterations resulting from neonatal exposure to diesel exhaust were not transient, but persisted throughout the lifetime of the rat. The first experiment clearly associated neonatal diesel exhaust exposure with decreased adult spontaneous locomotor activity. The second experiment indicated that the exposure of neonates to diesel exhaust produced differences in the acquisition of a relatively simple bar pressing task when the animals were 15 months of age.

The present study was designed to assess the effects of diesel exhaust on the development of the nervous system as measured by somatosensory and visual evoked potentials (SEPs and VEPs, respectively), collected from rat pups on days 28, 35, 42, and 49 postconception (pc).

METHODS

EXPOSURE

A general description of the exposure situation and the average levels of diesel exhaust components are reported elsewhere (2). The exposure system is described in detail by Hinners et al (3). Briefly, the diesel exhaust animals were exposed for eight hours per day, seven days per week to diesel exhaust generated by a Nissan CN 6-33. The particulate matter concentration was maintained at approximately 6 mg/m^3 by dilution with filtered air.

ANIMAL CARE

Sixty pregnant Sprague Dawley rats were ordered from the Michigan Charles Rivers animal supply laboratory. Fifteen dams were bred on each of four consecutive days. This design provided four experimental days to examine each tested day of pup development.

On day one after parturition, each litter was culled to eight pups, leaving four, five, or preferably six males per litter. Litters containing less than four males, or less than eight total pups, were excluded from the study. In addition to matching the litters for number and sex of pups, the following parameters were matched: day of conception, length of gestation, beginning time of parturition, number and weight of the pups per litter, and ratio of males to females. Of the initial 60 pregnant rats, 41 litters were matched and randomly placed into either the control or the exposed group.

ELECTROPHYSIOLOGY

General Procedure. The development of the SEP and the VEP was monitored from postconception day 28 or 35, respectively, through day 49. Rat pups are more maturationally similar compared to the time of conception than compared to the time of parturition. Therefore, the evoked potentials were evaluated in reference to conception as a means of reducing data variability.

When the pups reached the designated age of testing, they were anesthetized with pentobarbital at doses of 30 mg/kg (day 28 pc), 40 mg/kg (day 35 pc) or 50 mg/kg (days 42 and 49 pc). They were then placed in a stereotaxic apparatus modified to accommodate small animals, the skull was exposed, and silver ball electrodes were placed on the skull. Electrical brain activity was amplified by a Textronix 26A2 Differential Amplifier (0.1 to 10 KHz, 3dB Band width) and then sent to four inputs: 1) oscilloscope, 2) Nicolet 1170 signal averager, 3) Nicolet spectrum analyzer, and 4) multitrack tape recorder for data storage.

Somatosensory Evoked Potential. The SEP was elicited by 1 mamp pulses of 0.5 msec duration, delivered to the tibial nerve of the left hind limb at the tibale. Evoked potentials were recorded from an active electrode located on the skull above the somatosensory projection area, 2mm lateral to bregma. The reference electrode was 6mm anterior to the active electrode. The stimuli for day 28 pc were presented to the pups in three sets of 128 single pulses. The data included the averaged peak latencies and amplitudes from the first and third sets of stimuli. On days 35, 42, and 49 pc, 128 double pulse stimuli, with interstimulus intervals of 300, 200, and 150 msec, respectively, were presented and averaged at a rate of 0.5 Hz. The data consisted of latencies and amplitudes of the various peaks following each stimulus of the double pulse paradigm. The frequency spectrum of all electroencephalograms was monitored in order to investigate the level of anesthesia.

Visual Evoked Potentials. The visual system matures more slowly than the somatosensory system. Therefore, VEPs were not obtained on day 28 pc, and longer ISIs were employed for the double pulse paradigm on days 35 and 42 pc (800 and 300 msec, respectively). By day 49 pc, the visual system could respond to the same ISI (150 msec) being used for the SEPs. Dark adaptation of the pups was accomplished during the time the SEPs were being elicited. The VEPs were recorded from above the visual projection area of the right hemisphere (2mm lateral, and 6mm posterior to bregma). Placement of the reference electrode was 6mm anterior to the active electrode.

RESULTS

SOMATOSENSORY EVOKED POTENTIALS

Figures 1, 2, and 3 are examples of SEPs elicited on days 28, 35, and 42 pc, respectively. A day 49 pc response has not been included because of its similarity to the day 42 pc SEP. The most prominent feature of the evoked potential on day 28 pc was a negative peak labeled N2. On all days except day 28 pc, the evoked potential waveforms consisted of a series of positive and negative going peaks. The peaks were labeled, beginning at the stimulus artifact of the first pulse as P11, N11, P21, etc., and beginning at the second stimulus artifact as

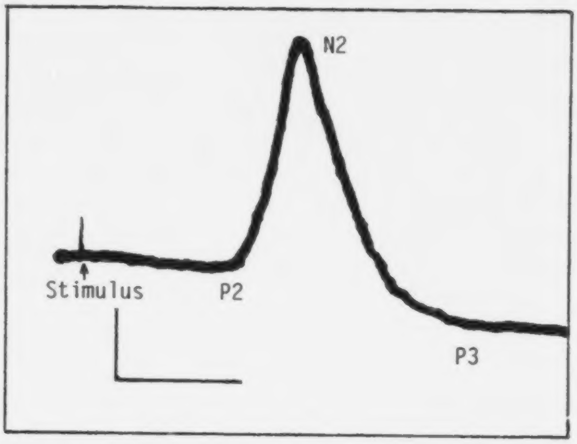

Figure 1. Typical SEP from a day 28 post conception, male rat. Calibration is 10 microvolts and 50 milliseconds.

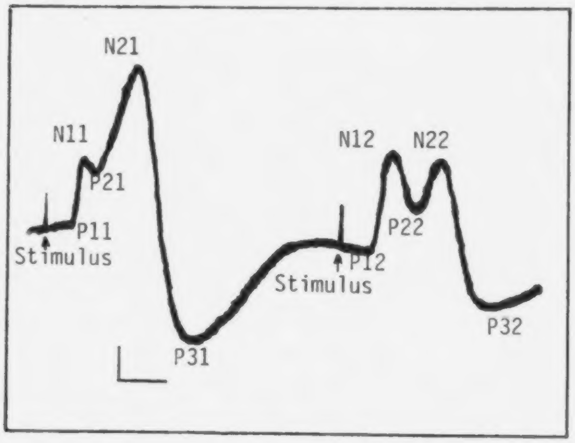

Figure 2. Typical SEP from a day 35 post-conception, male rat. Calibration is 20 microvolts and 50 milliseconds.

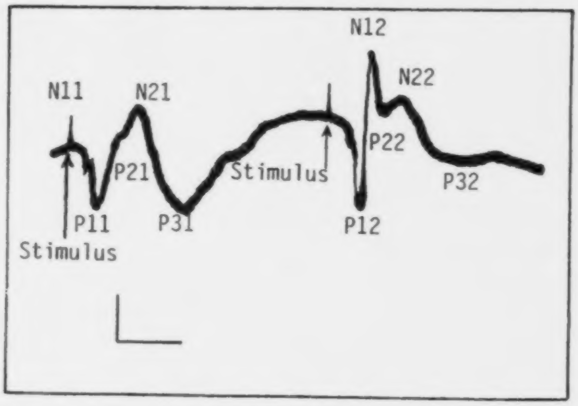

Figure 3. Typical SEP from a day 42 postconception, male rat. Calibration is 20 microvolts and 50 milliseconds.

P12, N12, etc. Tables 1 and 2 are summaries of the peak latency and amplitude data, respectively, obtained from the four days tested. On the days when the double pulse paradigm was employed, the peaks of the second evoked potential were usually slower and of different amplitude than the corresponding peaks of the first response.

The most significant differences between the control and exposed groups manifested themselves on day 35 pc. Figure 4 consists of group composite waveforms produced from all SEPs taken on day 35 pc; i.e., 1792 double pulse SEPs per group (128 averages per animal x 14 animals per group). The top waveform is the response to the first stimulus of the double pulse paradigm; the bottom waveform is the response to the second stimulus. The boxes around each peak indicate the standard error of the mean for both latency and amplitude. A comparison between the control composite and the diesel exhaust exposed composite reveals two major differences. First, all latencies to the various peaks are significantly longer for the diesel exhaust exposed group. Second, the difference between the N2 latency in the response to the first stimulus and N2 latency in the response to the second stimulus is significantly longer in the diesel exposed group than in controls. This relationship also exists for P3 latencies. These results are an indication of slower electrophysiological recovery in the central nervous system of neonatal rats exposed to diesel exhaust as compared to control animals. Day 35 pc is the only experimental day in which consistent statistically significant differences are detected.

Table 1. Latencies to Various Peaks of Somatosensory Evoked Potentials Taken From Male Rat Pups on Days 28, 35, 42 and 49 Postconception.

Day PC	Exposure Condition	Response	P1	N1	P2	N2	P3
28	Control	1st 128 Single Pulses			62.7 ± 1.3	86.1 ± 1.0	140.2 ± 3.9
	Exhaust				61.6 ± 0.8	86.0 ± 1.5	138.3 ± 3.7
	Control	3rd 128 Single Pulses			66.0 ± 1.5	88.6 ± 7.0	144.9 ± 4.9
	Exhaust				66.0 ± 2.1	87.3 ± 1.2	141.5 ± 5.2
35	Control	1st of Paired Pulses	22.9 ± 0.7^A	34.3 ± 1.0^A	45.1 ± 1.6^B	83.4 ± 2.1^A	131.8 ± 3.5^A
	Exhaust		25.0 ± 0.6	38.0 ± 0.9	51.8 ± 1.0	88.9 ± 1.6	143.1 ± 3.2
	Control	2nd of Paired Pulses	24.5 ± 0.8^A	42.0 ± 1.4^A	63.3 ± 2.9^B	88.3 ± 4.1^B	130.6 ± 6.1^C
	Exhaust		28.3 ± 0.9	47.1 ± 1.2	74.4 ± 2.1	104.8 ± 3.3	155.4 ± 3.
42	Control	1st of Paired Pulses	22.2 ± 0.7	41.4 ± 2.8		56.1 ± 3.2	92.9 ± 2.7
	Exhaust		20.9 ± 0.4	43.0 ± 2.4		57.8 ± 5.9	93.4 ± 2.5
	Control	2nd of Paired Pulses	23.0 ± 0.8	33.1 ± 1.1	41.4 ± 1.1	53.7 ± 1.1	93.4 ± 3.5
	Exhaust		22.2 ± 0.5	32.3 ± 1.0	38.9 ± 1.0	51.2 ± 1.0	87.6 ± 2.7
49	Control	1st of Paired Pulses	21.8 ± 0.4	30.3 ± 0.7^A	36.8 ± 1.1	54.0 ± 1.1	93.9 ± 4.3
	Exhaust		22.2 ± 0.6	34.1 ± 1.4	39.3 ± 1.1	55.5 ± 1.6	94.6 ± 4.3
	Control	2nd of Paired Pulses	25.5 ± 0.8	34.5 ± 0.7	42.6 ± 1.7	57.1 ± 1.7	107.0 ± 5.2
	Exhaust		26.8 ± 1.4	36.1 ± 1.0	42.0 ± 1.7	61.0 ± 2.5	110.0 ± 5.4

A= $P < 0.050$
B= $P < 0.010$
C= $P < 0.005$

Table 2. Amplitudes of Various Peaks of Somatosensory Evoked Potentials Taken From Male Rat Pups on Days 28, 35, 42 and 49 Postconception.

Day Pc	Exposure Condition	Response	P1	N1	P2	N2	P3
28	Control	1st 128 Single Pulses			-0.6. ± 0.8	-23.4 ± 2.8	5.8 ± 0.9
	Diesel				0.8 ± 0.4	-20.4 ± 2.9	5.6 ± 1.2
	Control	3rd 128 Single Pulses			0.3 ± 0.4	-26.6 ± 4.9	5.5 ± 1.4
	Diesel				0.7 ± 9.2	-29.7 ± 5.6	7.2 ± 1.5
35	Control	1st of Paired Pulses	0.5 ± 0.7	-25.9 ± 2.0	-18.4 ± 2.0	-64.8 ± 6.9	45.8 ± 2.8
	Diesel		0.2 ± 0.6	-28.6 ± 1.8	-17.5 ± 1.9	-60.0 ± 5.1	46.8 ± 4.0
	Control	2nd of Paired Pulses	1.5 ± 1.1	-32.2 ± 4.5	-10.8 ± 3.4A	-33.5 ± 5.0	28.2 ± 2.9
	Diesel		2.4 ± 0.7	-28.4 ± 2.0	-3.0 ± 2.7	-25.8 ± 3.8	29.3 ± 3.9
42	Control	1st of Paired Pulses	20.9 ± 3.2	-19.9 ± 5.4		-14.1 ± 4.4	28.4 ± 4.8
	Diesel		26.2 ± 3.4	-31.6 ± 16.3		-17.7 ± 8.9	37.1 ± 5.9
	Control	2nd of Paired Pulses	26.4 ± 7.0	-20.0 ± 6.3	-5.7 ± 2.3	-14.3 ± 3.0	13.7 ± 2.1
	Diesel		38.3 ± 5.5	-19.5 ± 5.5	-7.4 ± 3.2	-21.0 ± 4.0	23.9 ± 5.1
49	Control	1st of Paired Pulses	72.3 ± 12.1	-7.0 ± 6.0	-7.0 ± 6.0	-46.6 ± 8.0	21.8 ± 4.1
	Diesel		80.0 ± 10.0	-6.9 ± 8.9	-2.2 ± 6.2	-43.9 ± 6.0	19.0 ± 5.1
	Control	2nd of Paired Pulses	78.6 ± 8.0	-23.3 ± 8.8	-5.7 ± 5.7	-28.9 ± 5.4	26.9 ± 7.4
	Diesel		82.4 ± 12.3	-16.3 ± 12.9	-1.4 ± 5.8	-27.7 ± 6.6	16.8 ± 3.7

A= $P < 0.05$

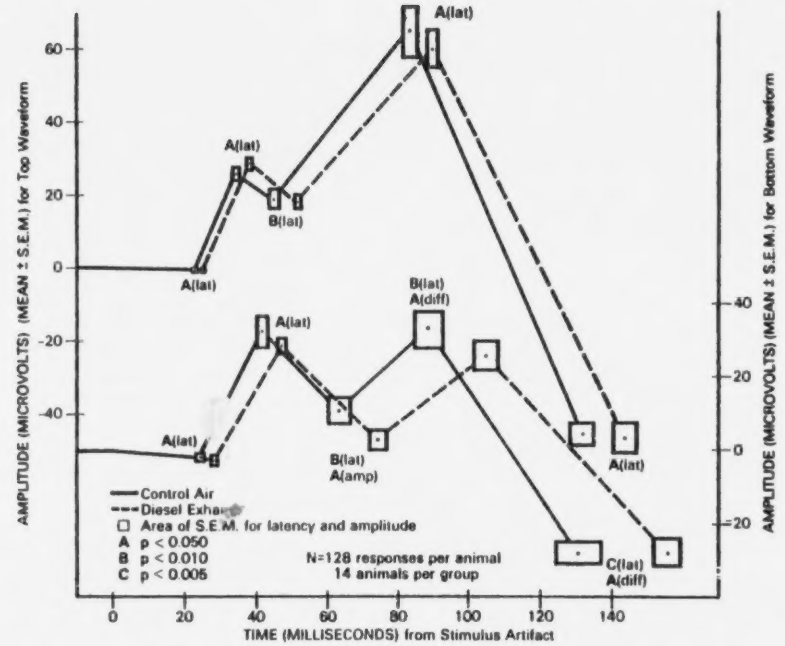

Figure 4. Group composite waveforms of day 35 post conception (pc) double plus somatosensory evoked potentials from animals exposed to either control air or diesel exhaust from day 22 or 23 pc. Interstimulus interval between top and bottom waveforms is 300 msec. Double pulse delivered at a rate of 0.5 Hz.

VISUAL EVOKED POTENTIALS

The VEP waveforms are visually similar to the SEP waveforms already presented. Tables 3 and 4 are summaries of the VEP latency and amplitude data, respectively, obtained on days 35, 42 and 49 pc. On day 35 pc, the latencies of all VEP peaks occurring before 300 msec were greater than controls, but the differences reached statistical significance for only the P2 peak of the response to the first stimulus.

DISCUSSION

Significant differences between control and diesel exhaust exposed neonates were detected for developing SEPs and VEPs. The electrophysiological differences can be attributed to differences between the nervous systems of the two groups. The exhaust exposure may affect both the central and peripheral nervous systems (CNS and PNS). For example, the slower latencies to the various peaks may be an indication of decreased myelination in both the CNS and PNS. CNS effects can be hypothesized by regarding the recoverability differences. The ability to follow rapid stimulation is limited by synaptic phenomenon. Because all of the synapses involved with the SEP are located in the CNS, the recovery disparity between the control and exposed groups can be attributed to a lesion(s) in the CNS.

SEP alterations were found in the diesel exhaust exposed group on day 35 pc and not on other days of testing. This may be related to the extremely rapid rate of neural growth and development which occurs in the rat around day 35 pc (approximately day 14 postparturition) (4). Among the processes occurring prior to and during this period are cell migration, axon myelination, cell differentiation, synaptogenesis, and elimination and stabilization of neuronal connections (5). In particular, the rate of increase in rat brain weight peaks around day 10 postparturition, and the in brain cholesterol around day 16 postparturition (4). The rapid rate of

Table 3. Latencies to Various Peaks of Visual Evoked Potentials Taken From Male Rat Pups on Days 35, 42, and 49 Postconception.

DAY	Exposure Condition	Response	P1	N1	P2	N2	P3	N3
35	Control	1st of Paired Pulses	74.2 ± 3.4	102.4 ± 4.3	142.5 ± 4.5^C	166.7 ± 4.7	231.0 ± 5.8	355.5 ± 14.9
	Diesel		79.9 ± 3.5	110.7 ± 5.4	161.5 ± 4.0	179.7 ± 5.5	247.5 ± 10.1	351.4 ± 16.8
	Control	2nd of Paired Pulses	77.2 ± 3.3	110.8 ± 4.7	151.3 ± 16.1	169.5 ± 17.2	225.6 ± 6.6	337.6 ± 13.2
	Diesel		79.1 ± 3.1	113.8 ± 4.8	167.7 ± 12.4	198.3 ± 3.9	217.7 ± 15.1	334.4 ± 20.9
42	Control	1st of Paired Pulses	15.4 ± 3.2	24.8 ± 1.6	38.3 ± 1.2	51.4 ± 1.9	138.0 ± 4.7	193.8 ± 5.1
	Diesel		15.7 ± 2.1	24.9 ± 1.6	36.5 ± 0.6	48.7 ± 0.8	139.2 ± 4.7	195.5 ± 4.1
	Control	2nd of Paired Pulses	22.1 ± 2.3	32.2 ± 2.5	40.4 ± 1.6	59.3 ± 2.2	118.8 ± 6.0	183.0 ± 7.2
	Diesel		15.0 ± 4.4	29.9 ± 1.5	41.7 ± 1.0	59.7 ± 2.1	121.4 ± 5.8.	201.3 ± 6.1
49	Control	1st of Paired Pulses	13.2 ± 2.2	24.0 ± 0.6	33.6 ± 0.5	47.5 ± 0.7	92.7 ± 3.8	111.6 ± 6.7
	Diesel		14.1 ± 3.1	24.5 ± 1.0	34.3 ± 0.7	50.4 ± 2.8	93.3 ± 4.6	104.2 ± 6.8
	Control	2nd of Paired Pulses	25.1 ± 3.9	33.1 ± 0.6^A	43.4 ± 0.9	53.8 ± 0.8	94.7 ± 3.0	104.9 ± 7.7
	Diesel		31.1 ± 2.0	36.7 ± 1.5	44.5 ± 1.3	59.4 ± 3.5	97.9 ± 2.8	126.9 ± 8.5

A= $P < 0.05$
C= $P < 0.005$

Table 4. Amplitudes of Various Peaks of Visual Evoked Potentials Taken From Male Rat Pups on Days 35, 42 and 49 Postconception.

DAY	Exposure Condition	Response	P1	N1	P2	N2	P3	N3
35	Control	1st of Paired Pulses	3.3 + 1.0	-26.8 + 3.6	-5.4 + 2.5	-11.8 + 3.1	15.4 + 1.6	3.7 + 1.0^A
	Diesel		7.7 ± 2.8	-41.6 ± 14.9	-1.1 ± 2.1	-4.4 ± 2.4	19.1 ± 3.7	-0.1 ± 1.3
	Control	2nd of Paired Pulses	5.4 + 1.8	-24.8 + 5.2	-0.7 + 7.5	-3.86 + 6.1	16.0 + 2.4	-3.6 + 1.5
	Diesel		4.6 ± 2.2	-32.5 ± 8.1	5.5 ± 4.7	1.4 ± 7.6	14.3 ± 4.5	-2.6 ± 1.5
42	Control	1st of Paired Pulses	1.7 + 0.5	-0.2 + 0.7	23.4 + 4.1	-28.9 + 4.0^A	19.4 + 2.7	-12.8 + 2.5
	Diesel		2.3 ± 0.8	-0.6 ± 0.9	34.6 ± 6.5	-60.3 ±12.1	21.9 ± 6.1	-17.7 ± 3.1
	Control	2nd of Paired Pulses	2.8 + 2.7	-0.1 + 2.5	16.6 + 3.2	-28.9 + 6.4	8.3 + 3.2	-14.4 + 5.2
	Diesel		5.8 ± 2.9	-0.2 ± 2.5	18.4 ± 3.4	-27.7 ± 5.8	11.9 ± 4.5	-17.0 ± 3.5
49	Control	1st of Paired Pulses	2.0 + 1.1	-1.5 + 1.2	106.0 + 2.6	-79.1 + 19.2	26.5 + 7.9	-3.7 + 1.0^A
	Diesel		1.6 ± 1.0	-10.4 ± 8.7	58.1 ± 26.2	-91.0 ± 20.8	27.2 ± 7.4	0.1 ± 1.3
	Control	2nd of Paired Pulses	0.2 + 8.2	-13.8 + 10.4	81.8 + 26.7	-69.9 + 26.6	58.9 + 14.2	-3.6 + 1.5
	Diesel		9.4 ± 3.7	-6.0 ± 10.8	29.4 ± 13.3	-59.0 ± 13.1	38.3 ± 9.4	-2.6 ± 1.5

A= $P < 0.05$

neural growth is reflected in rapidly changing evoked potential waveforms recorded around day 35 pc. A toxic effect which alters or retards development would produce a larger absolute difference between control and exposed waveforms around day 35 pc than on the other days tested. Specifying the particular process(es) affected by diesel exhaust without further study of development under exposure conditions is difficult because each developmental stage is highly dependent on the successful completion of previous events.

It should also be realized that disruption to any of the growth processes may result in permanent sequale. Male siblings of the animals used in this experiment were monitored, as adults, for spontaneous locomotor activity (SLA). The results of the SLA experiment indicated that the adults, exposed to diesel exhaust as neonates, were significantly less active than control animals (2). Other behavioral experiments involving diesel exhaust exposed animals have been reported in a companion paper(2).

The electrophysiological data reported here suggests that the long-term behavioral alterations produced by neonatal diesel exhaust exposure are related to the failure to develop a normally functioning nervous system. The long-term nature of the behavioral aberrations demonstrates that: 1) developmental alterations are not countered during subsequent nonexposure periods; 2) neonatal rats are more sensitive than adults to diesel exhaust exposure; and 3) the effects in neonates are qualitatively different from the transient depression of activity produced in exposed adult rats (1, 2).

Most investigations of diesel exhaust toxicity have employed adult animal exposure. The investigations of possible developmental effects have used classical teratological endpoints and have not investigated the functional competence of the pups (6, 7). With the projected increase in the number of diesel-powered light duty passenger vehicles, a growing number of developing humans will be exposed to diluted diesel exhaust. In order to help assure the protection of these sensitive individuals, the effects of diesel exhaust on developmental processes should be investigated further.

ACKNOWLEDGEMENTS

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General Discussion

C. RUDD: You indicated on a slide that the rat's brain and the cholesterol levels are very imporatant on day 35. Did you read slides of any rat brains and do cholesterol levels in diesel exposed animals vary from controls?

D. LAURIE: No we didn't.

C. RUDD: In your report, were these changes on day 35 in all experiments which you did?

R. LAURIE: It only happened on day 35.

C. RUDD: How many experiments did you do?

R. LAURIE: This was the first experiment.

EFFECTS OF SIX-MONTH EXPOSURE OF RATS TO PARTICULATE CARBON AND NITROGEN DIOXIDE

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ABSTRACT

To evaluate the toxic effects of specific components of diesel exhaust, rats were exposed to carbon particulate matter, carbon particulate matter with adsorbed NO_2, or NO_2 alone. Exposure was 8 hours per day, 7 days a week, for 6 months. Animals were removed from the study every two months and evaluated for changes in pulmonary function, pulmonary alveolar macrophages, alveolar wall permeability and lung pathology. Other rats were examined for alterations in behavior (running wheel activity) and reproductive function. Although the data is still being analyzed, no obvious overt toxic effects were found. In one phase of the activity study a significant depression of activity occurred but was not replicated in the second phase. Before any definitive statement can be made, analysis of the data must be completed.

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INTRODUCTION

The data to be reported here result from a cooperative effort between the Department of Environmental Health at the University of Cincinnati and the E.P.A. This collaboration originally began during the TAME (Toxicological Assessment of Mobile Emissions) studies with a specific interest in sulfuric acid emissions resulting from the use of catalytic converts. With the increased use of diesel engines, attention has now been shifted to the health effects of diesel exhaust. Several studies already conducted by the EPA (1) have shown that exposure to diesel exhaust at various stages of development in the rodent result in definite neurobehavioral effects. Other studies have found a wide variety of toxic effects resulting from exposure to diesel exhaust (2).

Since the E.P.A. is conducting a very extensive research program on complete diesel exhaust, the studies described here were concerned only with selected components within the exhaust. Specifically, the study was designed to investigate what toxic effects would result from exposure to carbon particulate matter alone or carbon particulate matter in conjunction with gas NO_2. The hypothesis tested was that the gas adsorbed on respirable carbon particulate matter would reside in the lungs longer than the gaseous portion of the atmosphere and thus could potentially have a much more toxic effect. Parameters examined included measures of general growth, spontaneous locomotor activity, pulmonary function, pulmonary alveolar macrophages, lysozyme, permeability of the alveolar wall and lung pathology.

METHODS

Overall Experimental Design: The study consisted of two separate groups of rats which were run concurrently — one group at the University of Cincinnati, the other at the E.P.A. The 2 groups were initially drawn from the same sample pool. Limitations in facilities resulted in the necessity of the study being conducted at both institutions. A control group and a group exposed to NO_2 were run at the EPA, while a control group, a charcoal particulate only group, and a charcoal particulate with adsorbed NO_2 group were studied at U.C. Finally, the behavioral studies were divided into two separate phases - one involving adults, the other neonates born in the exposure chambers. The other parameters were collected on animals exposed from the beginning of the study.

Treatment Groups: From an initial pool of 350 fifty g male CFE rats from Charles River, five groups of 70 each were randomly chosen for each exposure condition. For each exposure condition, 10 rats were placed in 35 cm Wahmann running wheels, with the remaining 60 being placed in hanging wire cages. Every two month 20 rats were selected from each exposure condition; 10 were evaluated for pulmonary function and 10 used in assessing biochemical indices. In addition 12 females (225 g, same supplier) were included in each treatment condition for reproduction studies.

Exposure Conditions: Animals at both facilities were exposed for 8 hours per day, seven days a week. Total duration of the exposure was 6 months.

U.C.- Animals were housed in three $21m^3$ chambers. One treatment group received cocoanut dust pretreated with NO_2, as well as free NO_2 gas at 5.5 mg/m^3 and 2½ ppm., respectively. A second treatment group received 5 mg/m^3 of plain charcoal powder only. A third chamber contained a clean air control treatment group. Mass median aerodynamic diameter particle size of the dust in these chambers was between 3 - 4 μm. Free NO_2 gas was delivered from cylinders containing 0.5% NO_2 gas in air. Charcoal/NO_2 pretreatment was performed by mixing 100% NO_2 gas with charcoal powder to first yellow in a separatory funnel. Approximate concentration by weight was 10% NO_2 and 90% NO_2 + charcoal. Charcoal dust was generated by agitating a charcoal bed with air currents, producing a fluidized bed. The charcoal dust was then passed through a vertical elutriator where charcoal powders + free NO_2 gas were diluted with clean air flowing at approximately 2½ m^3/min.

Charcoal dust was sampled on Polyvic filters for eight hours and weighed. A Sinclair-Phoenix Smoke Detector was also used to obtain immediate approximations of dust concentration. Particle size was determined with an Anderson Impactor. NO_2 gas concentrations were determined manually with Saltzman Reagent (NIOSH P&CAM 108).

E.P.A.- Animals were housed in eight chambers, each having a volume of $3m^3$. The exposure facilities have been described indetail elsewhere in this symposium.(3) The control group was exposed to filtered air only, while the experimental group received 2½ ppm NO_2. The gas was obtained from the same supplier as that used by U.C. and generated in the same manner.

Parameters Examined

Behavior: Weanling rats were placed in Wahmann running wheels two weeks prior to commencement of exposure in order to obtain baseline data. They remained in the wheels continously for the duration of the study. Number of wheel revolutions was recorded daily. Food and water intake and body weight were recorded weekly. In the second phase of the study, weanlings from the reproduction study were placed in the wheels and maintained on the same schedule. However since most of the rats' activity occurs during nightime, and exposure would be greatest during such periods of exercise, the light-dark cycle was reversed. Thus, although exposure conditions remained the same, the effective concentration was increased.

Pulmonary Function

Every two months, 10 rats from each treatment were sacrificed and measurements made of the following pulmonary parameters: lung weight, carbon monoxide diffusion (D_LCO), vital capacity, alveolar volume and residual volume by gas dilution and water displacement. Methods used were modified from those of O'Neil et al. (4). The lungs were subsequently saved for pathological examination.

Biochemistry

As in the pulmonary function studies, 10 rats from each treatment were sacrificed every two months and the pulmonary alveolar macrophage examined. Albumin - ^{131}I (80 μ Ci/Kg body weight) was injected into the caudal vein of the experimental animals 6 hours before sacrifice. After the animals were sacrificed, the lungs were lavaged and aliquots of the lavage were used for macrophage counting, ^{131}I activity counting, and protein and lysozome determinations.

Reproduction

After one months of exposure, the 12 females in each treatment were mated with 12 males from the same treatment (males were subsequently used in studies of pulmonary function). Number of successful pregnancies as well as litter size and birth weights were recorded. On day 3, litters were culled to no more than eight pups. At weaning, 3 males per litter were saved; the rest were sacrificed. At 40 days of age, 10 male offspring per treatment were placed in the running wheels.

RESULTS

The general health stuatus of the experimental animals was unaffected by exposure to any of the treatment conditions. Food and water consumption was the same across all conditions as was also body weight.

Analysis of the activity data for the first group of rats placed in the running wheels showed that while there were no overall treatment differences, there was a significant interaction of Treatment X Time. ($F=1.63, df=30,405; p<.02$) Inspection of Fig. I. reveals that while the control group and the Carbon + NO_2 group started out equal, they showed the greatest separation during the period of highest activity (week 7). By the end of the study the groups were once again nearly equal. There were no significant differences found anywhere between the control group and the NO_2 group (Fig. 2).

In the second study, although the three groups again started out equal (see Fig. 3), the difference increased rapidly between the control and the exposed animals. However, analysis of the data revealed that the differences (Treatment alone) only approached significance ($F=2.92$,

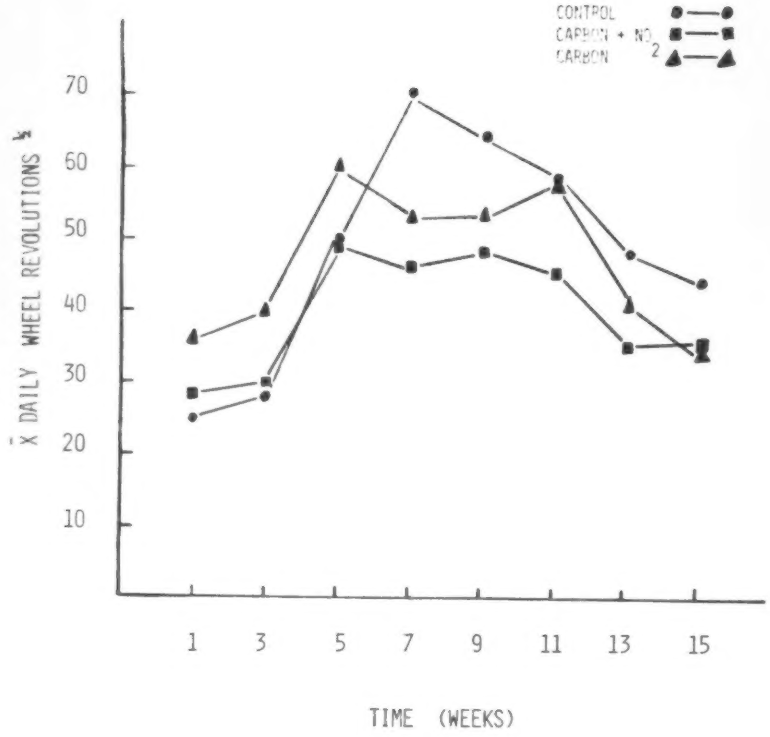

Figure 1. Running wheel activity of rats exposed to carbon particulate matter alone, particulate matter + NO_2, or filtered air. Exposure began on week 3 and continued for the duration of the study.

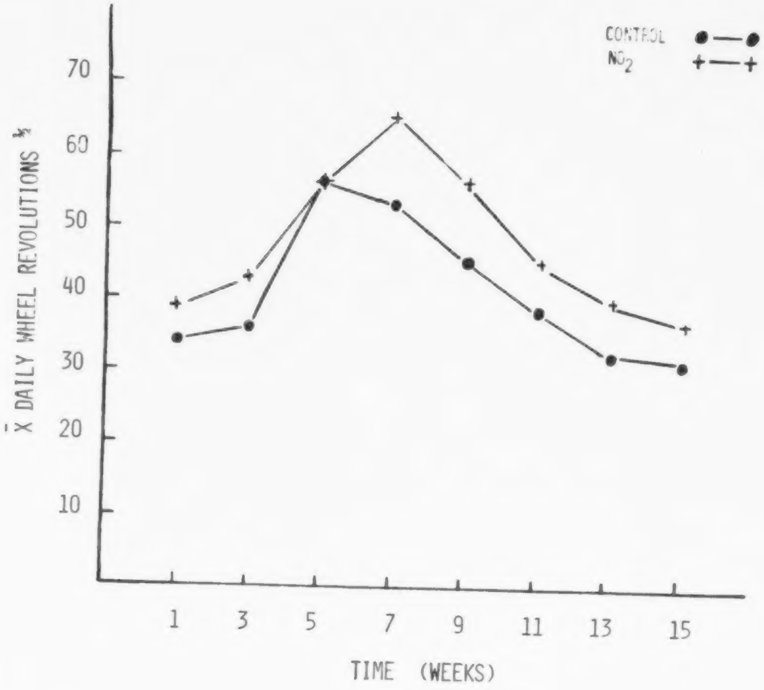

Figure 2. Running wheel activity of rats exposed to NO_2 or filtered air. Exposure began on week 3 and continued for the duration of the study.

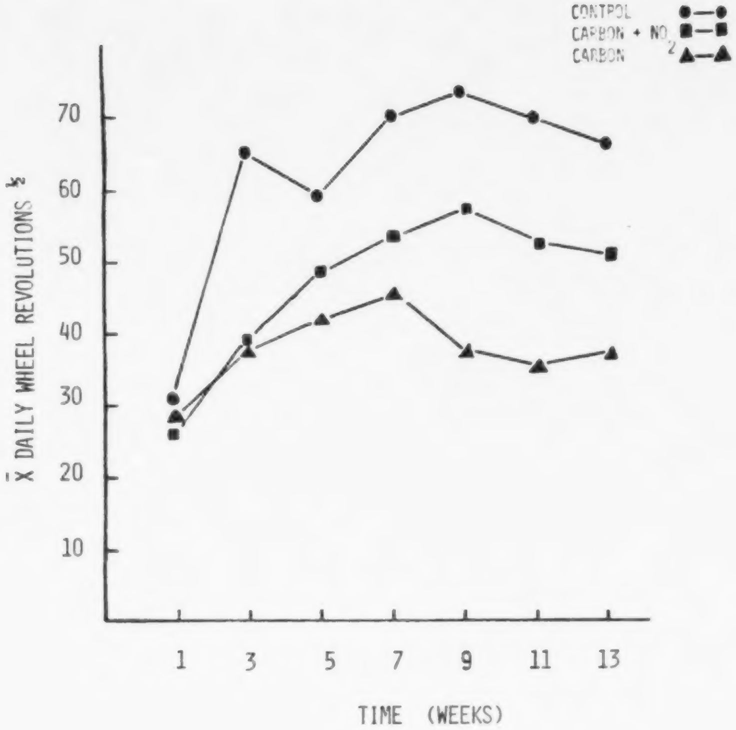

Figure 3. Running wheel activity of rats born in exposure chambers and exposed from birth on to either carbon particulate matter alone, particulate matter + NO_2, or filtered air.

df=2,26; p<.07). Unlike the first run where the groups had nearly equal activity levels at the end, the groups in this run still showed marked separation. It should be pointed out though, that the carbon only group showed the greatest reduction and not the carbon + NO_2 group as in the first run. There were no significant differences between the NO_2 and control group (Fig. 4).

In the reproduction phase of the study there were no significant differences found on any of the parameters measured (see Table 1). Rate of growth was also comparable across all three groups.

Tables 2 and 3 contain the results of the pulmonary function evaluation studies. These data are only preliminary in nature and do not include the results from those animals exposed for the full six months. Although there are significant differences in several of the parameters it is premature at this time to interpret them until results are available from the last study and from the pathology reports.

In previous studies involving sulfuric acid exposures (5), a drastic effect of inhaled $Al_2(SO_4)_3$ was observed on the pulmonary alveolar macrophage which resulted in 3 to 5 fold increases in leakage of lysozyme into the extracellular fluid. Moreover, aluminum sulfate caused a pronounced increase in the leakage of intravenously injected radiolabelled albumin into the alveolar space. These two parameters were highly correlated, therefore, it was hypothesized that damage to macrophages with consequent spillage of lysosomal enzymes in the alveolar space lead to an inflammatory process resulting in an increase in alveolar wall permeability.

In this study rats exposed to carbon showed only a slight but significant increase in extracellular lysozyme; however, during the first 4 months of exposure, the alveolar wall permeabiliy to radio labelled albumin remained unchanged. No effects were seen in the rats exposed to NO_2.

Finally, studies of lung pathology have thus far been done on a small sample from the two-month exposure group. In general, very few effects were seen-especially with the Carbon alone or NO_2

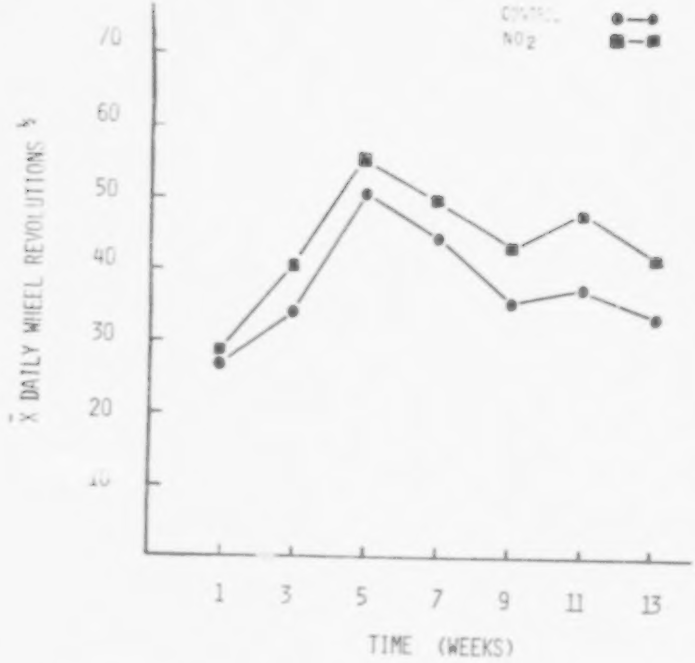

Figure 4. Running wheel activity of rats born in exposure chambers and exposed from birth on to either NO_2 or filtered air.

Table I. Reproductive parameters of rats mated in exposure chambers and exposed daily throughout the gestational period.

	# of Successful pregnancies	$\bar{x}$ litter size	$\bar{x}$ birthweight (male) g	$\bar{x}$ birthweight (female) g
Control	9/12	14.2	6.7	6.2
Carbon	7/12	13.5	6.5	5.9
Carbon + NO_2	9/12	13.8	6.8	6.2

Table 2. Pulmonary function parameters of rats exposed to carbon particulate matter alone, particulate matter + NO_2 NO_2 alone or filtered air for 8 weeks. Values indicated are mean ± standard diviation.

	Body Weight (g)	Lung Weight (g)	Residual Volume H_2O (ml)	Vital Capacity (ml)	Alveolar Volume (ml)	Residual Volume Ne (ml)	CO Diffusion $(mlCOmin^{-1}mmHg^{-1})$
Control	453	1.632	1.157	12.7	13.5	0.8	.171
	21	0.098	0.298	1.4	1.4	0.4	.031
Carbon	458	1.692	1.366	13.6	14.6	1.0	.199
	53	0.107	0.182	2.0	2.3	0.6	.073
Carbon + NO_2	482*	1.806*	1.490	13.7	14.8	1.4	.198
	37	0.142	0.364	1.6	1.0	1.0	.045
Control	426	1.673	1.476	11.9	12.9	0.9	.205
	37	0.139	0.424	2.0	2.1	0.4	.038
NO_2	423	1.727	1.426	12.6	13.3	0.7	.223
	9	0.085	0.553	1.7	1.8	0.4	.026

* Significantly different from appropriate control at $p<.05$

Table 3. Pulmonary function parameters of rats exposed to carbon particulate matter alone, particulate matter + NO_2, NO_2 alone, or filtered air for 16 weeks. Values indicated are mean ± standard diviation.

	Body Weight (g)	Lung Weight (g)	Residual Volume H_2O (ml)	Vital Capacity (ml)	Alveolar Volume (ml)	Residual Volume Ne (ml)	CO Diffusion ($mlCOmin^{-1}mmHg^{-1}$)
Control	556 37	1.973 0.200	1.258 0.362	16.0 1.2	17.6 1.5	1.6 0.8	.280 .051
Carbon	574 84	2.149 0.253	1.056 0.165	15.9 2.4	17.7 2.3	1.4 0.6	.302 .054
Carbon + NO_2	517* 30	2.101 0.161	1.112 0.178	15.8 2.1	16.6 2.5	0.8* 0.5	.272 .052
Control	534 48	2.265 0.456	1.678 0.393	16.2 1.4	17.5 1.7	1.3 0.6	.315 .025
NO_2	489* 29	1.979 0.111	1.357* 0.221	16.5 1.4	17.6 1.6	1.2 0.6	.267* .028

* Significantly different from appropriate control at $p<$

alone groups. In the NO_2 + Carbon group a few of the animals showed dilation of the peripheral airways, indicating a weakening of the pulmonary parenchyma or a loss of some alveolar septal strength. Again these results are very preliminary and whether this trend continues after longer exposure remains to be seen.

DISCUSSION

Since the data from this study are still being analyzed, any conclusions that are drawn at this time must still be considered tentative. With this in mind, it can be stated that no obvious, overt detrimental health effects were observed resulting from exposure to carbon particulate matter, NO_2, or a combination of the two. Growth curves and food and water consumption was comparable across the groups. There were no differences in any of the reproduction parameters, either. A few scattered measurements involving pulmonary function were significant, but they appear to be due to random statistical variation. Pathology also failed to detect any significant toxic effects.

Behavior, as measured by running wheel activity, was significantly depressed in the first run but not the second. This was unexpected since 1.) those in the second run had been exposed from birth and 2) exposure occurred, on a daily basis, during their most active period. While the results of the second run were not statistically different they followed the same trend as the first run and of previous studies (1), i.e., depression of activity. From the available data it does not appear that any dramatic affects can be attributed to any of the treatment conditions or that there was any significant difference between carbon particulate matter alone or particulate matter and NO_2.

However it would be premature to state that carbon particulate matter alone or in conjunction with NO_2 are without any toxic effects. This would be tantamount to proving the Null Hypothesis, which is clearly impossible. It is very easy to develop an experimental design with features which would make it difficult to obtain any significant differences. Unfortunately, many of these factors often occur in toxicological research--factors such as limitations in sample size, very low exposures, short duration of exposure, etc. Before any definitive statements can be made, greater attention must be given to these condiserations.

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General Discussion

S. DUTTA: What were the gross appearances of the lung? Do you see blackness of the lung and a lot of carbon particles?

L. HASTINGS: There is a definite grayish appearance to the lungs. They are not as dark as animals exposed to diesel exhaust but they are darkened.

S. DUTTA: What about lymph nodules in the lung or lymph nodes? We have just seen four weeks, as well as two weeks, exposure at 6,000 micrograms per cubic meter. That would be about six milligrams per cubic meter. That exposure to diesel exhaust causes a dense color change as well as clear blackening of the lymph nodes close to the trachea. I was wondering what kind of things you observed in the lymph nodes as well as in the lung? If they are clear, then it would appear to me that carbon particles are an entirely different species so far as diesel particles are concerned.

L. HASTINGS: I didn't do the gross pathology on these. Although I didn't see it, I am sure the lymph nodes would have contained particles; the macrophages were loaded with particles very similar to the diesel exposed animals and there were what looked like carbon particles adhered to the alveolar walls. They were very similar to the diesel exposed lungs.

SPEAKER: Does the answer lie in your particle size? I didn't hear you say particle size. What was the particle size?

L. HASTINGS: Three to four microns - not as small as the smallest diesel particles.

J. ORTHOEFER: Actually, they are quite a bit larger than the diesel particles.

L. HASTINGS: The lymph nodes were black.

RIGGER: In your further data collection activities, how much is your sample size going to increase in terms of the number of animals to each of the four specific categories?

L. HASTINGS: I think we will have to look at the data first and see what type of variability we are experiencing in order to determine what future size the samples should be.

RIGGER: I think the fact may be that you haven't found anything, any trends, or anything significant to date. It may be a function of numbers purely. The probability of erroneously rejecting some known, or accepting some known hypothesis is very high given the sample sizes you have right now.

SPEAKER: Did you have a chance to determine the methemaglobin concentration in the blood or nitrate since you have used nitrogen dioxide as your gas component?

L. HASTINGS: No.

ATMOSPHERIC COMPONENT CONCENTRATIONS IN THE ANIMAL EXPOSURE CHAMBERS, TADE II STUDY

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ABSTRACT

The atmospheric data from the various analyses - daily monitoring of major pollutants and intermittent measurements of other components in the animal exposure chambers - was used to calculate whole study averages for the first year of operation of the diesel engine. The concentration values, for exhaust emissions diluted by clean air (16 to 18X), served as reference data in the evaluation of animal health effects and as a guide for controlling the engine system operation.

The atmospheres in the animal exposure chambers were analyzed for both gaseous and particulate components in each of twelve such chambers supplied with atmospheres containing diluted engine exhaust emissions and of four reference chambers supplied with clean air (C.A.). Daily monitoring prevailed for the components of major concentration - carbon dioxide (CO_2), carbon monoxide (CO), total hydrocarbons (THC), nitric oxide (NO), nitrogen dioxide (NO_2) and sulfur dioxide (SO_2). The atmospheres also were checked for levels of ammonia gas (NH_3) and ammonia products resulting from chamber animal contributions interacting with the other atmospheric components. Analytical procedures and instrumentation are identified in the report by R. Hinners(1).

RESULTS AND DISCUSSION

The chambers were monitored for temperature and relative humidity as factors involved in the overall animal health effects, whether directly or as a result of interactions in the atmosphere.

The concentration values were used as reference data relating to animal health effects and as a guide for controlling the engine system operation within limits defined for the study.

The engine conditions coupled with the level of dilution air volume were used to maintain a suspended particulate concentration of about 6 mg/m^3 in the animal exposure chambers. The weekly averages, as shown in the figure, calculated as a 52-week average of 6.39 ± 0.78 mg/m^3.

Average values of the component concentrations for (1) the exposure chambers supplied with diluted exhaust emissions and (2) the chambers supplied with clean air, were used to establish composite weekly averages for each of the two atmosphere types.

A study average of each of several major components for the first 52 weeks showed:

Table I. - Component concentrations

	Exhaust Emissions (E.E.) Chambers	Reference (C.A.) Chambers
CO_2, %	0.29 ± 0.03	0.05 ± 0.01
CO, ppm	19.72 ± 2.13	2.07 ± 0.53
THC, ppm as C	7.84 ± 0.99	3.49 ± 0.38
NO, ppm	11.23 ± 1.53	0.08 ± 0.03
NO_2, ppm	2.65 ± 0.55	0.05 ± 0.03
SO_2, ppm	2.06 ± 0.43	0.05* ± 0.01
Particulate, mg/m^3	6.39 ± 0.78	- - -

* Based on clean air values obtained at beginning of the day.

Initial samples of the particulate mass in the C.A. atmosphere chambers were barely measurable (0.1 mg/m^3) and such measurements were discontinued.

From the above table, it is evident that there was a considerable contribution, percentagewise, of hydrocarbons (HC) to the exhaust emissions chambers, 7.84 ppm, from the background clean air, 3.49 ppm. The difference, 4.35 ppm, represents the actual input from the engine exhaust emissions.

The sulfur dioxide (SO_2) value for the reference chambers was determined from measurements made on the clean air supply before the sampling operation was locked into the cycling program at the beginning of the day's run. During the cycling program, the exhaust emissions chambers (n=12) with their relatively high pollutant concentrations were sampled sequentially before the group of reference chambers (n=4). The SO_2 monitoring instrument was very slow in responding to the large drop-off of concentration from the last exhaust emissions chamber to the nearly zero concentration of the first and

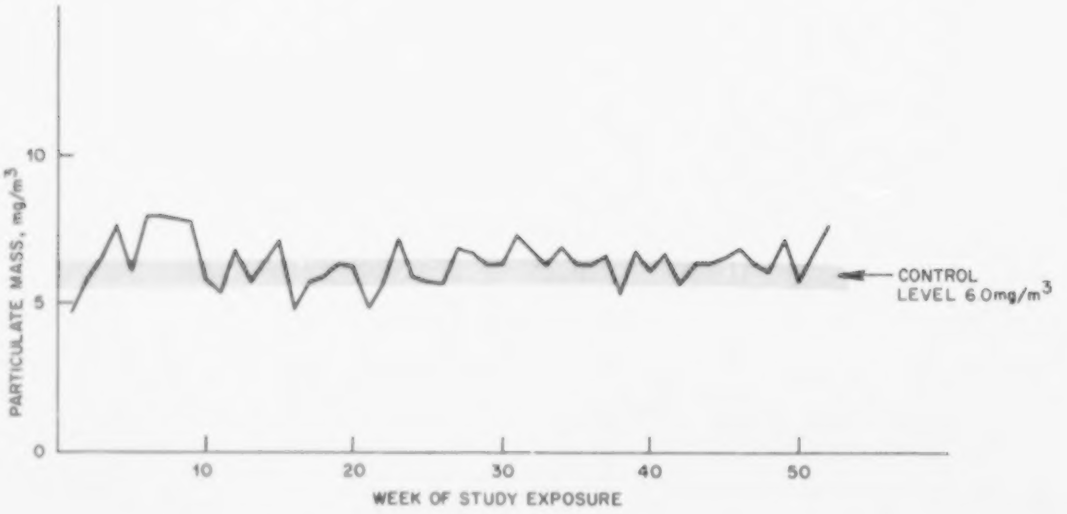

Figure 1

Weekly Average Concentration of Diesel Particulate in Exposure Chamber Atmospheres, Tape 2

succeeding reference chambers so that falsely high levels of SO_2, averaging about 0.54 ppm, were recorded for those chambers.

The particulate mass value was determined from the samples collected on teflon-coated filters. The weights that were measured for similar samples on glass fiber filters were invariably higher by about 1 mg/m^3; they are judged to be less accurate because of the considerable sensitivity of that filter material to humidity effects.

The particulate matter in filter samples examined by a scanning electron microscope revealed an apparently basic unit of 0.05-0.10 um size, with agglomerates reaching almost 1.0 um in diameter.

Sulfate concentrations, as determined by ion chromatography applied to the aqueous extracts of the filter samples, averaged 1.33 mg/m^3 during the first quarter (3 months) of the study. It represented 23.0 percent of the total-particulate concentration measured over the same interval. A more thorough study of the ion components in the particulate was done during a period immediately preceding the start of the presently reported study; the procedure of that type analysis is described in a report by N. P. Barkley(2).

Ammonia gas (NH_3) concentrations were measured in the various exposure chambers primarily to minimize if not eliminate episodes of high levels of the gas. Since the concentration varied with the number and kind of animal in the chamber and with the cleaning schedule for the chamber, there resulted quite a large variation in NH_3 concentration among the chambers. Although the average value for the entire study was only 0.62 ppm inclusive of all the chambers, there did occur certain daily episodes in a chamber that measured as much as 11.3 ppm NH_3.

The DNPH method (gas chromatography of the dinitrophenylhydrazine derivatives of the aldehydes) for 5 to 6 individual aldehydes was applied to the exposure chamber atmosphere samplings. However, when considerable difficulty was encountered in obtaining reliable or reproducible results, that method was set aside in favor of several other aldehyde procedures - for total, aliphatic aldehydes(3), for formaldehyde(4), and for acrolein(5). The study averages of those components by the latter group of procedures showed 0.18 ppm aliphatic aldehydes, 0.11 ppm formaldehyde and a little less than 0.03 ppm acrolein.

Hydrocarbon concentration measurements by means of the heated (350°F) sampling line were made as daily checks on (the same) two exposure chambers throughout the study. They averaged 0.75 ppm higher than the ambient temperature values (average of 7.60 ppm) monitored in all twelve of the exhaust emissions chambers over the corresponding period of time.

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PULMONARY FUNCTION CHANGES IN CHINESE HAMSTERS EXPOSED SIX MONTHS TO DIESEL EXHAUST

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ABSTRACT

Chinese hamsters were exposed for eight hours per day to automotive diesel exhaust emissions which were diluted with air (18 to 1) and had a particulate level of 6.4 mg/m^3. Pulmonary function measurements were made after six months exposure. Body weight (BW), lung weight (LW), vital capacity (VC), residual volume by water displacement (RV_W) and by gas dilution (RV_D), alveolar volume (V_A), and carbon monoxide transfer factor (D_LCO) were measured. LW showed a significant increase in the diesel exposed animals (P .01) while VC, RV_W, and D_LCO showed decreases (P .01). Static deflation volume-pressure curves showed depressed deflation volumes for diesel exposed animals when volumes were corrected for body weight and even greater depressed volumes when volumes were corrected for lung weight. However, when volumes were expressed as percent vital capacity, the diesel exposed animals had higher lung volumes at 0 and 5 cm H_2O. Results of the pathological examination of the lung tissue will be necessary for final analysis of our findings. However, preliminary interpretation indicates possible emphysematic changes which are compatible with the observed decrease in D_LCO.

INTRODUCTION

The effects of exposure to diesel exhaust upon the lungs cannot be accurately predicted from the results of previous studies using either individual pollutants or whole emissions from gasoline engines. A portion of the gaseous pollutants, for example, are adsorbed onto the surface of the particulate matter (Stokinger, 1973), altering both the depth of penetration (Task Group on Lung Dynamics, 1966) and residence time in the lungs (Creasia et al, 1971). There is also little information concerning the composition of sulfates present in diesel exhaust. Certain sulfates, such as zinc ammonium sulfate, have been found to be considerably more irritating than sulfuric acid, the primary form in catalyzed automobile exhaust (Amdur, 1970). Finally, diesel exhaust contains a wide range of both aliphatic and aromatic compounds, the concentration of which, and even the chemical structure, are not well defined (Karasek et al, 1974).

The present study was designed to evaluate the effects of 6-months exposure to a concentration of diesel exhaust containing 6 mg/m^3 particulate upon pulmonary function in the Chinese hamster.

METHODS

General Procedure. Adult male Chinese hamsters were exposed to diesel exhaust eight hours per day seven days per week at a dilution ratio of approximately 1:18. Particulate concentration averaged 6.4 mg/m^3. The animals were exposed in stainless steel wire cages 11 inches square. Nine or ten animals were housed per cage. Food and water were provided ad libitum. Total length of exposure was 6 months. Following completion of exposure, the animals were removed, weighed, and body temperature measured via rectal probe. The hamsters were then anesthetized with pentobarbital sodium administered intraperitoneally. The trachea was exposed and cannulated just below the glottis.

Vital Capacity. The animal was placed in the supine position on an insulated platform and the tracheal cannula was attached to the breathing port of the D_LCO apparatus (see Figure 1). The animal was hyperventilated, with the respiratory valve in the "B" position, for a period sufficient to produce apnea lasting 10-15 seconds. With the valve in position "A" fresh air was injected into the lungs of the animal from a calibrated syringe until the pressure in the airway plateaued at +25 cm H_2O. The volume reached was defined as total lung capacity (TLC). Air was then withdrawn into the syringe until the pressure in the airway plateaued

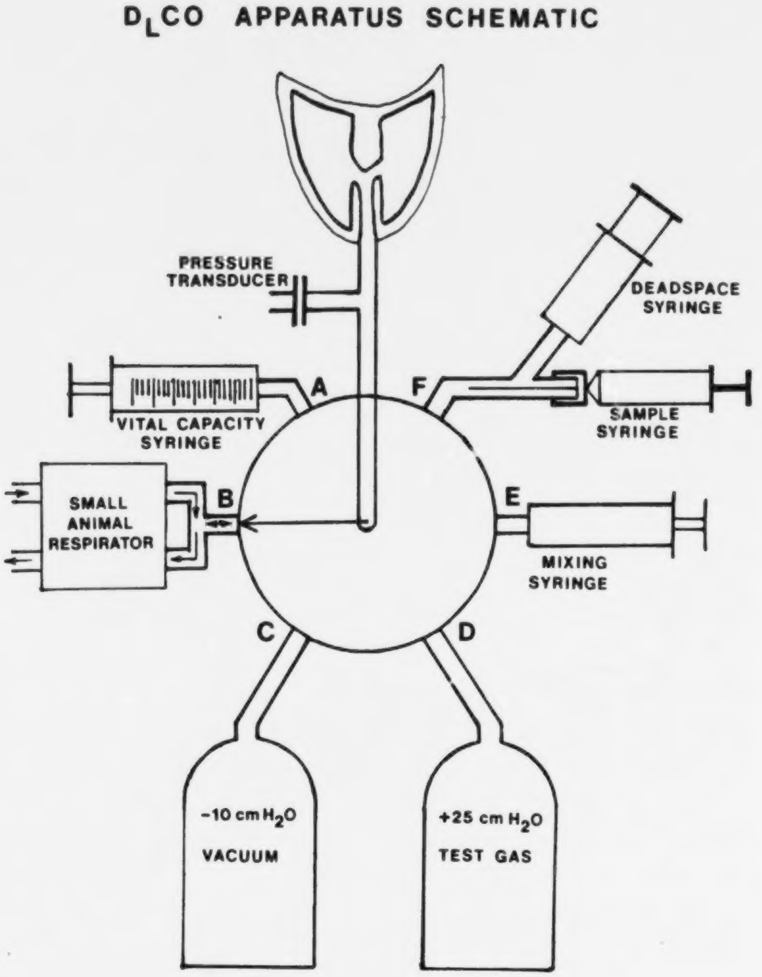

Figure 1. D$_L$CO Apparatus Schematic.

at -10 cm H_2O. The volume reached was defined as residual volume (RV). The corrected difference between the two syringe readings was defined as vital capacity (VC). The animal immediately returned to breathing fresh air.

Alveolar Volume. The animal was again hyperventilated until apnea was produced. The valve was returned to the "C" position allowing the lungs to be deflated to RV. The valve was turned to the "D" position allowing the lungs to be inflated quickly to TLC with air containing a known quantity of the inert gas neon. The valve was turned to the "E" position and the gas in the lungs was pumped in and out ten times to facilitate even distribution of the neon within the lungs. The valve was turned to the "F" position allowing passive expiration of about one-third of the vital capacity into a preset ground glass syringe, effectively eliminating the anatomical and mechanical deadspace air. The remaining alveolar air could then be sampled into a gas-tight chromatography syringe for analysis. The animal returned to breathing fresh air.

D_LCO. The animal was hyperventilated until apnea was produced. The valve was turned to the "C" position allowing deflation of the lungs to RV. The valve was then turned to the "D" position allowing rapid inflation of the lungs to TLC with air containing known amounts of neon and carbon monoxide. Approximately 8-10 seconds after inflation, the valve was turned to the "F" position and the alveolar gas sampled as described above. During all of the above procedures, the body temperature was monitored and an attempt made to keep it as close to the preanesthesia level as possible by use of a variable intensity heat lamp mounted above the platform. The procedures and calculations for determination of D_LCO, alveolar volume and vital capacity as described above are modified from those reported by O'Neil et al (1977).

Static Deflation Volumes-Pressure Curves. The lungs were exposed by opening the chest cavity and retracting the ribs. The lungs were connected in parallel, by way of the tracheal cannula, to a syringe and a pressure transducer. Pressure was monitored on an oscilloscope and volume read directly from the syringe. The lungs were given a known volume history by inflating twice from 0 to 30 cm H_2O, maintaining for five seconds, and then deflating in increments of five cm H_2O, and maintaining at each subsequent pressure for 15 seconds. Deflation was taken to -10 cm H_2O. Volumes were recorded at each pressure decrement.

Final Lung Treatment. The lungs were then removed and attached to a pressure reservoir of -10 cm H_2O. Lung weight and displacement volume were determined. This volume was defined as residual volume (RV_W) as compared with the above-mentioned determination by gas dilution (RV_D).

Finally, the lungs were instilled with 10% buffered formalin and maintained at 30 cm H_2O while submerged in a bath of the same fixative. After 36 to 48 hours they were stored in individual jars of buffered formalin to await preparation for pathological examination.

RESULTS

Our preliminary results are presented in Table 1 and Figure 2. The apparent difference in RV, as determined by water displacement, may not be real since correction was not made for tissue density. The diesel exposed animals had lungs that were mainly coal black in color. The added density of the deposited material made the lungs denser, but without knowing the weight and density of deposited material a total correction for lung density cannot be made.

The static deflation volumes, Figure 2 are plotted graphically against pressure in four different ways: A) percent vital capacity, B) absolute volume of air removed from lung, C) lung volume corrected for body weight, and D) lung volume corrected for lung weight. Where differences between diesel and control animals exist at least at the P=.05 level this is indicated with an asterisk.

DISCUSSION

Pathological investigation of the lung tissue has not yet been performed. However, the elevated lung volumes at 0 and 5 cm H_2O for the volume-pressure curve where volume is expressed as %VC indicate a possible emphysematic condition in the diesel exposed animals. This is consistent with the significantly reduced D_LCO found in these same animals. Exposure at the same concentration for longer periods of time and exposure at higher concentrations may be necessary to more carefully evaluate the potential effects of exposure to diesel exhaust on lung function.

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Table 1

Effects of Diesel Exhaust Exposure Upon Pulmonary Function Parameters in Chinese Hamsters

	BODY WEIGHT (G)	LUNG WEIGHT (MG)	LW/BW (MG/G)	VITAL CAPACITY (ML)	VC/BW (ML/G)	VC/LW (ML/G)	RV_W (ML)	RV_D (ML)	V_A (ML)	D_LCO	D_LCO/VA
	CONTROL										
X	36.0	227	6.35	.96	.0268	4.25	.319	.36	1.17	.0029	.0027
S.D.	4.1	22	.54	.10	.0038	.60	.131	.18	.31	.0011	.0005
N	9	9	9	9	9	9	8	5	5	9	9
	DIESEL										
X	35.2	374	10.62	.75	.0217	2.07	.016	.40	1.16	.0015	.0015
S.D.	3.4	55	1.17	.12	.0046	.54	.025	.13	.17	.0008	.0006
N	10	10	10	10	10	10	9	8	8	10	10
T	NS	$P \leq .01$	$P \leq .01$	$P \leq .01$	$P \leq .05$	$P \leq .01$	$P \leq .01$	NS	NS	$P \leq .01$	$P \leq .01$

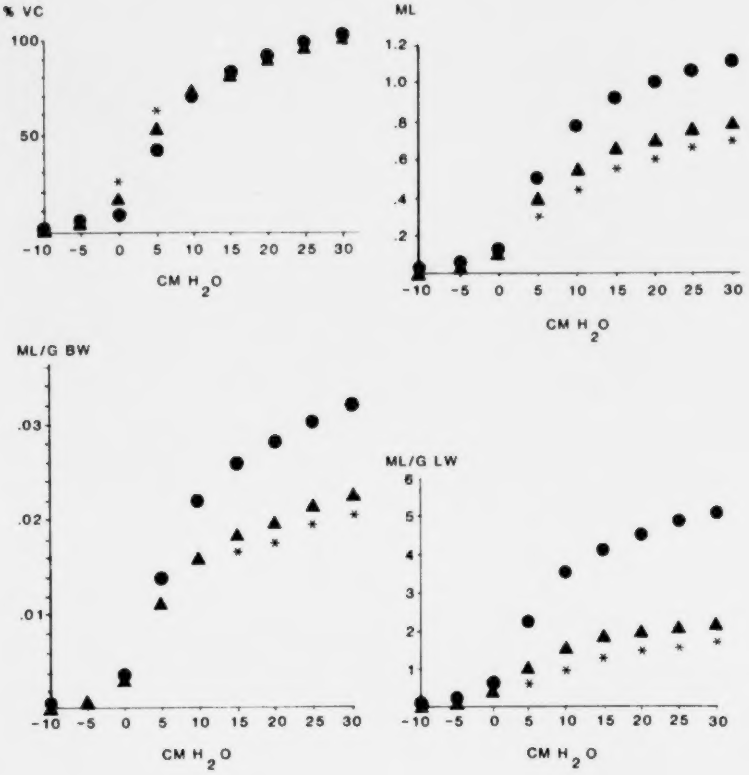

Figure 2. Static Deflation Pressure Volume Curves Controls are indicated by circles and diesel exposed are indicated by triangles. Asterisks indicate significance at least at the $P = .05$ level.

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PULMONARY FUNCTION EVALUATION OF CATS AFTER ONE YEAR OF EXPOSURE TO DIESEL EXHAUST

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ABSTRACT

Adult male, inbred, disease-free cats of uniform age and size were exposed eight hours per day, seven days per week to a 1:18 dilution of diesel exhaust emissions. After one year of exposure, the animals were removed from the chambers for measurement of lung volumes, forced expiratory flow rates, dynamic compliance and resistance, diffusing capacity, and nitrogen washout. No important changes in pulmonary function were detected with the exception of a decrease in closing volume ($P < .05$). The inability to detect decrements in pulmonary function may have been due to insufficient concentration of exhaust, insufficient exposure length, or to the use of a species resistant to diesel exhaust.

To test these possibilities, the cats are being exposed for an additional year, and another species, hamsters, are being exposed for future testing at exhaust dilutions of 1:18 and 1:9.

INTRODUCTION

The use of diesel engines has increased greatly in the past 30 years. Much of this increase has been due to a changeover to use of diesel engines in locomotives, heavy trucks, construction equipment, and farm tractors. With increasing fuel costs, the percentage of diesel engine equipped automobiles is also increasing and is expected to continue to increase in the foreseeable future.

Diesel exhaust contains a wide variety of pollutants, many of which are known to cause lung damage (4). Moreover, many of these pollutants are adsorbed on the very fine carbon particulate allowing for effective penetration into the deep lung (8). For these reasons, it was considered important to study the effects of chronic inhalation of diesel exhaust emissions upon lung function.

The present experiment was designed to evaluate the effects of chronic diesel exhaust inhalation upon pulmonary function and pathology in cats.

METHODS

Young adult male cats, born and maintained in a disease free environment and inbred for several generations were purchased from Liberty Labs, Liberty Corners, New Jersey. The cats were uniform in size and varied in age by less than two weeks. The cats were exposed in chambers 1.38 meters square with an interior volume of 2.83 cu m. Each chamber contained a wire mesh floor and two half shelves of similar material at 2/3 and 1 1/3 meters above the floor. This allowed the cats about 4.7 m^2 (50 sq. ft.) of horizontal space. Eight or nine cats were housed per chamber. They were not caged but allowed to roam free. Food and water was provided ad libitum. The cats were exposed eight hours per day, seven days per week for one year to diesel exhaust diluted to produce a particulate concentration of 6 mg/m^3. Details of the exposure conditions have been presented elsewhere in the proceedings by Hinners et al.

Prior to pulmonary function testing, the animals were fasted for one day. The testing followed 18-20 hours of no diesel exposure. The animals to be tested were anesthetized with Ketaset Plus (Ketamine 100 mg, Promazine 7.5 mg per ml) at a dose of 42 mg/kg. Following the induction of anesthesia, an esophageal balloon was placed in the lower third of the esophagus and an 18-22F endotracheal tube was inserted into the trachea with the aid of a laryngoscope. The cuff of the endotracheal tube was inflated and excessive length of the distal end trimmed even with the end of the mouth. The animal was then placed into the chamber, ventral side up, for compliance and resistance testing. For all other tests, the animal was in the prone position, dorsal side up.

Pulmonary mechanics were obtained from simultaneous volume, flow, and transpulmonary pressure tracings displayed on a twelve-channel photographic recorder (Electronics for Medicine, DR-12). Airflow through the pneumotachograph was measured with a differential transducer and electrically integrated to produce a volume trace. Dynamic pulmonary compliance (CL_{DYN}) was calculated from simultaneous volume and transpulmonary pressure tracings at points of zero flow (5). Average flow resistance ($RL_{Ave.\ Flow}$) was calculated from the change in transpulmonary pressure (at equal volumes) divided by the sum of inspiratory and expiratory flow.

All mechanics were measured while the animal was breathing spontaneously through the pneumotachograph only. The animal was inflated for ten seconds initially and periodically throughout the testing to expand atelectatic areas.

The pulmonary function tests requiring breathing maneuvers lung volumes, maximum expiratory flow-volume curve (FEF), diffusing capacity ($DL_{C^{18}O}$), nitrogen washout (ΔN_2), and closing volume (CV) were performed using a variable pressure plethysmographic chamber. The basic method employed was similar to that used in an external tank respirator; however, a hydraulic control system enabled the operator to bring about inspiration, expiration, breath holding, and breathing rate within the anatomical and physiological limits of the animal. Both flow and volume were controlled secondarily by changes in the pressure surrounding the animal. Inspiratory and expiratory airflow could be controlled from very low rates to the maximum within each subject. Likewise, volume could be controlled for both maximum inspiration and expiration. Inspiratory capacity (IC) was obtained by rapid depressurization to -70 cmH_2O from testing tidal position. Prior to the flow-volume testing it was determined that plethysmograph pressures of +70 cmH_2O would be used to produce maximal expirations. Inspection of flow-volume curves at increasing driving pressures showed that flow limitation characteristics had been reached at volumes above 50% Total Lung Capacity (TLC) when the plethysmograph pressure was greater than 70 cmH_2O. Therefore, FEF at 50% and 40% of TLC are values take at an effort-independent zone of the flow-volume curve. The curves were highly reproducible in each animal and demonstrated a low coefficient of variation (2-2.5%) in the effortindependent zone.

To ensure that sufficient intrathoracic driving pressure was developed, esophageal pressure was recorded during forced expirations. A trans-chest-wall pressure gradient was observed; however, intrapleural pressures of 30-35 cmH_2O were achieved which are efficient to produce flow maxima. A volume error, as a result of thoracic gas compression, was calculated to be approximately 3% at 50% TLC with the intrapleural pressure of 30-35 cmH_2O. This error was considered to be irrelevant because the results were compared in animals tested at the same driving pressures.

Breathing manipulations could be performed in anesthetized animals because of the apnea produced on inflation as a result of the inflation reflex documented by Hering and Breuer (3). The inspiratory inhibition had been demonstrated by recording action potentials from the phrenic nerve.

IC and forced vital capacity (FVC) were recorded during a maximum inspiration, followed by a maximum expiration. Flow and volume tracings were recorded, which provided the essential data points for calculating forced expiratory flows and volumes ($FEV_{0.5,1.0}$) and peak expiratory flow (PF). This procedure of maximum inspiration followed by maximum expiration was performed initially and thereafter for all test maneuvers, insuring equal volume and flow histories.

The methods of Brashear et al (1) and Mitchell et al (6), were combined to obtain values for $DL_{C^{18}O}$ and TLC. The calculations for DLCO were performed according to the method described by Wagner et al (9), for $C^{18}O$. Gas analyses were done using a respiratory mass spectrometer (Perkin-Elmer MGA1100).

Distribution was studied using the single-breath nitrogen washout and closing volume according to the methods described by Buist and Ross (2).

RESULTS

The pulmonary function results for exposed and control cats are summarized in Tables 1 and 2. No significant differences were found in mechanical properties, diffusing capacity, uniformity of distribution, or ventilatory performance. Closing volume (volume of Phase 4) was found to differ from the control values; however, this change cannot be interpreted as impairment. The exposed cats demonstrated a lower closing volume than the controls indicating improved function of small airways. This effect is believed to be a Type 2 statistical error or some unexplainable adaptation phenomona. The latter explanation is unlikely based on our experience. It is probably due to high animal to animal variation seen in some of the pulmonary function tests.

A preliminary study was conducted by the EPA in which several animal species were exposed for 20 hours per day for up to two months to a similar concentration of exhaust as used in the

Table 1

Lung Volumes and Forced Expiratory Flow Rates in Cats Exposed to Diesel Exhaust

	N	EXPOSED MEAN	S.D.	N	CONTROL MEAN	S.D.
LUNG VOLUMES						
IC (ML)	21	278.7	44.81	21	301.9	49.56
FRC (ML)	21	158.2	35.61	21	265.4	42.22
ERV (ML)	21	69.2	24.58	21	67.0	19.05
RV (ML)	21	86.1	36.99	21	104.1	37.67
TLC (ML)	21	415.3	56.02	21	449.5	74.49
RV/TLC (%)	21	20.26	6.936	21	22.72	5.896
DYNAMIC LUNG VOLUMES						
FVC (ML)	21	348.4	43.46	21	368.9	42.14
FEV 5/FVC (%)	21	84.3	8.44	21	81.6	6.39
FEV1/FVC (%)	21	97.6	1.96	21	97.3	1.74
PEFR (ML/5)	21	1016.5	185.10	21	1041.8	174.17
FEF 50% (ML/5)	21	728.0	195.62	21	661.1	160.43
FEF 25% (ML/5)	21	490.4	186.81	20	481.4	199.49
FEF 10% (ML/5)	19	196.5	107.35	20	222.2	156.82
FEF 50%/FVC (FVC/SEC)	21	2.101	.5434	21	2.072	.4249
FEF 25%/FVC (FVC/SEC)	21	1.400	.4806	20	1.314	.5310
FEF 10%/FVC (FVC/SEC)	19	.569	.3131	20	.605	.4294
FEF 40% TLC (ML/5)	21	486.3	252.64	21	557.2	248.05
FEF 40% TLC/TLC (TLC/SEC)	21	1.185	.6355	21	1.317	.6368

Table 2

Dynamic Compliance and Resistance, Diffusion Closing Volume and Nitrogen Washout in Cats Exposed to Diesel Exhaust

	Exposed			Control		
	N	Mean	S.D.	N.	Mean	S.D.
MECHANICS						
($CMH_2O/L/5$)	21	10.675	4.5785	21	10.323	4.4305
(ML/CMH_2O)	21	23.536	7.2045	21	23.698	9.2548
DIFFUSION						
DLCO (ML STPD/ MIN/MM HG)	21	1.179	.3143	21	1.217	.3023
DLCO/VA (1/MIN/MM HG)	21	.00345	.00125	21	.00335	.00084
CLOSING VOLUME AND WASHOUT WITH N_2						
CV	21	25.6*	13.44	21	36.8	16.00
CV/VC (%)	21	7.91	3.276	21	10.46	4.6
(CV+RV)/TLC(%)	21	26.83**	6.993	21	20.05	7.192
$\%N_2/25\%VC$	21	.32	.206	21	.29	.302

* Significantly different from Controls $P<.05$

** Significantly different from Controls $P<.01$

present study (8). This initial study was designed to provide preliminary data on tolerance levels, toxic effects, and target organs. Some of the findings included detection of the presence of black granular particles in the alveolar macrophages, black pigment in the bronchial and carinal lymph nodes, increased pulmonary flow resistance, increased lung weight/body weight ratios, and sinus bradycardia in guinea pigs.

In the present study, in which animals were exposed for a longer period of time (one year), but only eight hours per day, convincing evidence is present that inhalation of diesel exhaust under these conditions does not result in functional alterations in the lungs of cats. This is in contrast to the preliminary study in which some positive responses were noted. We believe cats in the exposed groups have functionally adapted to this exposure. Increasing the concentrations of diesel exhaust or increasing the duration of this study may produce significant chronic pulmonary disease; however, the present results cannot provide a guide to those parameters which may be early indicators of functional alterations.

Minimal or no responses would be expected to result from the individual gaseous pollutants at the concentrations present. The particulate level might be expected to produce impaired ventilation as a result of the site of deposition and potential tissue response. The effect of the combination of gaseous pollutants, interaction with, or adsorption to the particulate, however, cannot be predicted.

The results reported here are from an ongoing study in which cats are being exposed for a planned two years to diesel exhaust emissions. Shortly after the start of the second year of exposure, the dilution of exhaust was decreased to produce a particulate concentration of 12 mg/m^3. Pulmonary function, pathology, and biochemical parameter will be assessed following completion of exposure. In addition, lung pathology is being evaluated from several cats that died during the year either from unrelated causes or during testing.

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APPENDIX

(Abbreviations)

IC	Inspiratory Capacity
FRC	Functional Reserve Capacity
ERV	Expiratory Reserve Volume
RV	Residual Volume
TLC	Total Lung Capacity
FVC	Forced Vital Capacity
PEFR	Peak Expiratory Flow Rate
FEF	Forced Expiratory Flow
RL	Resistance
Cl	Compliance
L.F.	Low Frequency
H.F.	High Frequency
DLCO	Diffusing Capacity
VA	Alveolar Volume

FUNCTIONAL AND MORPHOLOGICAL CONSEQUENCES OF DIESEL EXHAUST INHALATION IN MICE

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The mice used in this study were exposed for three months to light duty diesel exhaust and were supplied by the Health Effects Research Laboratory, USEPA, Cincinnati, OH. All of the animals were anesthetized i.p. with sodium pentobarbital (50 mg/kg body weight, Abbott Laboratories). We adapted pulmonary function methods used with other small laboratory animals to measure lung volumes and the single breath diffusing capacity for carbon monoxide (2). The data were analyzed by covariance analysis using $\log_{10}$ body weight as the covariate to account for weight differences between the two groups. Other animals were tracheostomized, the lungs were removed and then inflation fixed with 2% gluteraldehyde at 25 cm H_2O using a low resistance system (1). The fixed lung tissue was prepared for light and electron microscopy using standard techniques.

The results of the pulmonary function testing are summarized in Table 1. No statistically significant differences ($p > 0.05$) were observed between the two groups.

TABLE 1. PULMONARY FUNCTION OF MICE EXPOSED FOR 3 MONTHS TO LIGHT DUTY DIESEL EXHAUST*

	Control	Exposed
Number of animals	5	9
Body weight, g	25 ± 0.8	30 ± 1.1
Lung weight, g	0.15 ± 0.002	0.23 ± 0.025
Vital capacity, ml	0.89 ± 0.05	0.97 ± 0.04
Total lung capacity, ml	1.29 ± 0.05	1.40 ± 0.08
Residual volume, ml	0.39 ± 0.03	0.43 ± 0.07
Diffusing capacity for carbon monoxide, and CO absorbed/min x $torr^{-1}$	0.0174 ± 0.001	0.0178 ± 0.003

*Mean ± S.E.

Light and electron photomicrographs of parenchymal tissue from mice following diesel exhaust inhalation for three months are shown in Figures 1, 2, and 3. The alveolar macrophages accumulated carbonaceous material and appeared to be larger when compared to macrophages from the control animals (Figure 1). The macrophages became heavily laden with this material and they appear globular with the absence of pseudopodia (Figure 2). Diesel exhaust particles are also present in the interstitium, presumably in the lymphatics (Figure 2). The macrophages accumulate this carbonaceous material in discrete vesicles which appear to be membrane limited (Figure 3). Although no statistically significant functional differences were observed between these two groups of animals, it is clear that the diesel exhaust particles were accumulated in the lung parenchyma. Further investigations of pulmonary function and morphology would provide useful information on the potential toxicity of diesel exhaust.

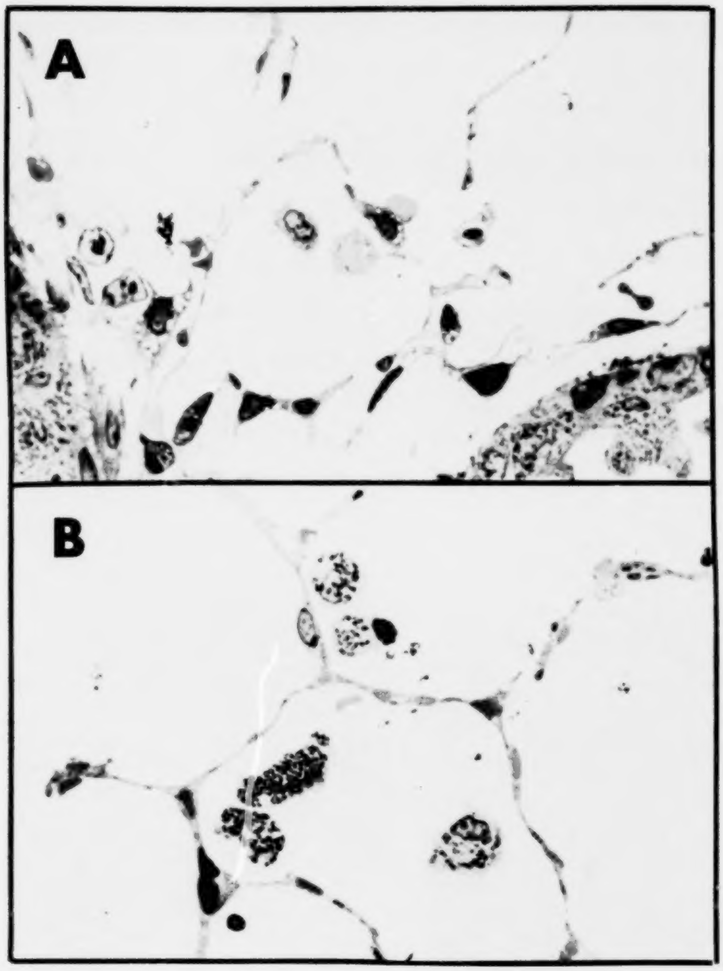

Figure 1. Photomicrographs of alveoli from a control animal (A) and an animal exposed to diesel exhaust (B). The alveolar macrophages from the exposed animals appear to be increased in size and to contain many carbonaceous particles. (1,175X)

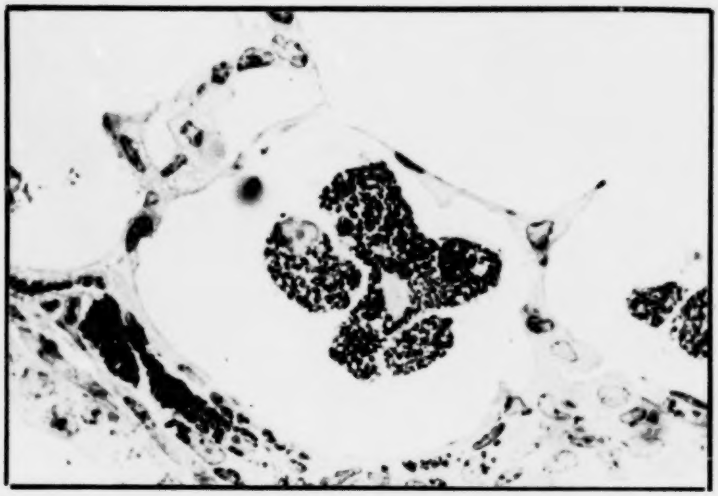

Figure 2. Photomicrograph of alveolar tissue from an animal exposed to light duty diesel exhaust. The alveolar macrophages are heavily loaded with carbonaceous particles, they appear to be globular in shape with a notable absence of pseudopodia. The particulate material is also evident in the interstitium, presumably in lymphatic vessels. (1,175X)

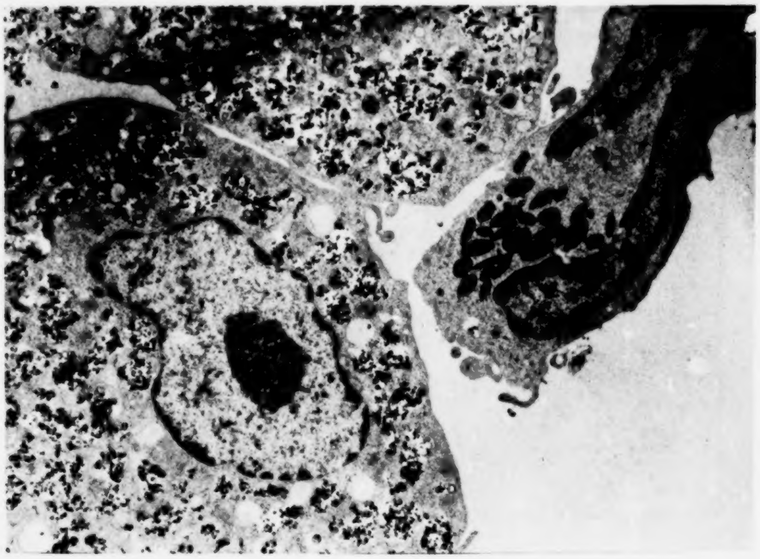

Figure 3. Electron photomicrograph of alveolar macrophages join the lungs of an animal exposed to diesel exhaust. Carbonaceous material appears to be confined in discrete membrane limited vesicles within the macrophage. The eosinophil, frequently seen in the alveolar space, does not appear to be phagocytizing any particles. (15,000X)

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ENCHANCED SUSCEPTIBILITY TO INFECTION IN MICE AFTER EXPOSURE TO DILUTE EXHAUST FROM LIGHT DUTY DIESEL ENGINES

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ABSTRACT

A series of experiments was conducted in which groups of mice were first exposed for various durations to diluted exhaust from light duty diesel engines and then briefly to an infectious aerosol generated by nebulizing cultures of a bacterial pathogen (Streptococcus). Typically, post-infection mortality was significantly greater in groups exposed to exhaust than in their corresponding control groups exposed to purified air only. Data of recent diesel and of past diesel- and catalyst-treated gasoline engine exhaust experiments suggest a somewhat greater excess mortality from (enhanced susceptibility to) bacterial infection in mice exposed to diesel exhaust than in those exposed to catalytic gasoline exhaust. Limited data on acute tests of NO_2 and acrolein vapor alone suggest that the infectivity-enhancing effect of diesel exhaust could be accounted for in large part by these components. Exposures to diesel exhaust, NO_2, or acrolein did not enhance the mortality response to a viral pathogen (A/PR8-34).

INTRODUCTION

In recent years there has developed an increasing awareness and concern about the potential adverse effects of environmental contaminants on the immune system and ability to resist infection. A number of contaminant materials have been demonstrated to impair various specific functions of the immune system and, significantly, host resistance to microbial pathogens, which depends on the integrity of several immunologic and physiologic components performing in concert.

Exposure to automotive exhaust prior to the era of the catalytic converter, and to certain of its component or resultant pollutants such as nitrogen dioxide (NO_2) and ozone, was shown to enhance susceptibility to infection by inhaled Streptococcus and Klebsiella pathogens.[1,2,3] More recently we have investigated the toxicity of catalyst-modified automotive gasoline-engine exhaust, and diesel engine exhausts, with respect to impairment of host resistance.[4,5,6] This report concerns recent similar tests of diesel engine exhaust.

METHOD

The experimental model ("infectivity test") used was adapted from that described by Coffin and Blommer[3] in connection with their early auto exhaust studies and later employed by others for evaluating various airborne contaminants. A series of experiments was conducted in which mice were exposed to diluted diesel-engine exhaust (test) or to purified air (controls) for brief, intermediate, or prolonged periods, shortly after which they were briefly exposed to an atmosphere containing airborne respirable pathogen in culture and then observed for response to infection. Mortality was the primary response by which group effects were compared. A greater incidence of mortality after exposure to infectious material among test mice compared to controls was considered to represent impaired resistance to the infectious process by whatever specific mechanism(s) responsible.

Young adult (usually 4 to 8 weeks), female, CR/CD-1[*†] albino mice were used. Purina Laboratory Chow and tap water were provided ad libitum. The basic group size of n=20 (e.g., 20 controls, 20 test) was used in individual experiments owing to limitations of the inhalation chamber used for infectious challenge.

The mice were housed in groups of 10 in wire mesh cages for their exposure to purified air (control) or diluted exhaust (test) atmospheres. Exposures were conducted using stainless steel and glass inhalation chambers previously described by Hinners, et al.[7,8] Chamber atmospheres were generated and distributed also as described by Hinners, et al.[8] Briefly,

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[*] Charles River Breeding Laboratories, Inc., Wilmington, Mass.

[†] Mention herein of a commercial product does not constitute endorsement by the Environmental Protection Agency.

exhaust was generated using alternatively one of two Nissan CN-6 diesel engines operated on a dynamometer stand providing for cycled operation ("Federal Short Cycle") consisting of a repeated series of acceleration, deceleration, cruise, and idle modes. Each day the engine was operated for exhaust generation and distribution for 8 hours, after an initial warm-up period during which exhaust was diverted. The fresh exhaust was mixed and diluted (overall first-year average dilution factor about 18:1) with carbon- and particulate-filtered and temperature-humidity conditioned air and distributed to mixing chambers and then to the inhalation chambers. The inhalation chambers were ventilated at the rate of about 15 volumes per hour, being positively exhausted and operated under conditions maintaining chamber pressure at about 0.3-0.4 in water, negative. Chamber atmosphere conditions were controlled at very near 72°F and 50% relative humidity. Engine performance was monitored and maintenance and adjustments were conducted so as to maintain the design level particulate at 6-7 mg/m^3 in the test-exposure chambers. Chambers and cages were serviced (cleaning, feeding, watering) on a frequent schedule to maintain general sanitary conditons, minimize ammonia production from animal excreta, and observe status of animal health.

Several times daily the chamber atmospheres were automatically sampled and analyzed for carbon dioxide (CO_2), carbon monoxide (CO) total hydrocarbons (HC), nitrogen oxides (NO, NO_2), and sulfur dioxide (SO_2). Chambers were also sampled manually at least daily for determination of total suspended particulates (TSP), and at periodic intervals for other components of interest (e.g., aldehydes, other organics, ammonia). Methods used and other engineering details are described elsewhere in this symposium.

A few experiments involving exposure to nitrogen dioxide (NO_2)-or acrolein (AC)-contaminated atmospheres were conducted. These are irritant constituents of automotive exhaust and were used in single-contaminant experiments to investigate the degree to which they might contribute to exhaust toxicity in terms of impaired resistance.

Immediately after control or test exposure, the mice were removed and transported. They were then placed in individually compartmented mesh cages and exposed to infectious aerosol for a brief period in a smaller inhalation chamber of comparable design. The infectious aerosol was generated by nebulizing an appropriate dilution of cultured pathogen in a DeVilbiss nebulizer (Mod. 40), the discharge of which was passed through a section of glass or Tygon tubing (to permit settling out of too-large droplets) and then into the chamber's air supply inlet. The pathogen culture strength, dilution, nebulizer operation, and chamber operation parameters, and exposure duration (generally 15 and 30 minutes for bacterial and viral aerosol challenges, respectively) were such as to yield a low positive morality response (5-20 percent) in control subjects so as to optimize detection and quantitation of enhanced response in test-exposed mice. In two experiments the mice were challenged with an enteric bacterial pathogen by intragastric intubation.

Three pathogens were used. The primary pathogen used for challenge in most of the experiments was a B-hemolytic group C Streptococcus pyogenes originally isolated from an animal lesion. It was used in the studies of Coffin[3] and later by Gardner, et al.,[9,10] from whom we obtained our stock culture. Active culture in Todd-Hewitt broth was freeze-dried in aliquots for stable storage. These were reconstituted and diluted as needed for infectious challenge exposures. The pathogen of next importance was a mouse-adapted A/PR8-34 influenza virus. It was passed in mice, titered, grown in brain heart infusion broth, frozen in aliquots, and thawed and diluted as needed for challenge exposures. The third pathogen, used in two experiments, was a Salmonella typhimurium. It was grown in trypticase soy broth, lyophilized, and resonstituted just prior to use. The additional viral and enteric pathogens were used to investigate whether diesel exhaust affected resistance in terms of these infectious phenomena as it was known to in the case of streptococcal pneumonia.

After infectious exposure the mice were housed for observation in plastic shoe-box cages in an animal room supplied with single-pass filtered, conditioned air. Body weights were recorded daily, and mortality and morbid signs were monitored, for at least two weeks, or for a sufficient additional period to accommodate recovery from the infection response (at least a week beyond the last death, with trend of increasing weight gain in surviving mice).

A total of 22 experiments were performed, including 13 involving acute (one 2-hour, the rest 6-hour), 7 subacute (two 8-day, four 15-day, one 16-day, 8 hours/day), and 2 chronic (307 and 321 days, 8 hours/day) exposure periods preceding infectious challenge. The Salmonella pathogen was used in two subacute (15-day)diesel exhaust (DE) experiments, the virus in four acute (one DE one NO_2, one with De and AC, and one with De, NO_2, and AC) and one 8-day subacute (DE, NO_2, and AC) experiments, and the streptococcal pathogen was used in all other experiments (all test exposure durations and agents).

Mortality following infection was the primary criterion used to assess whether resistance to infection was impaired by test exposure. Group mortality data was analyzed by the chi-square (uncorrected) technique for comparison between and among experimental groups. Other lethality data (e.g., time to death), and body weight changes relative to controls as a general index of morbid response to infection, were also examined.

Protocol and design features of this work are summarized in Appendix 1.

RESULTS AND DISCUSSION

The average concentrations for the primary exhaust constituents monitored in exposure chambers are summarized in Appendix 2 for several typical experiments. The design exhaust concentration for the period covered by these experiments was that providing chamber atmosphere total suspended particulate loading of 6 to 7 mg/m^3. The average concentration over the first year of operation was 6.4 mg TSP/m^3, as shown for Experiment CB-1,2. The corresponding average concentration of NO_2, a respiratory irritant gas and likely contributor to the impaired resistance to infection effect, was 2.8 ppm, comparable to the level in the experiments with NO_2 exposure (2.5 ppm). Concentrations of other constituents less regularly assayed (including aldehydes) should be available in another report in this symposium.

Data on the mortality response to infection (the primary measure of effect) for most experiments involving exposure to diesel exhaust are summarized in Appendix 3. Exposures of all durations to diesel exhaust resulted in enhanced susceptibility (impaired resistance) to lethal effects of bacterial infection. This was qualitatively consistent throughout the experiments, was clear cut and statistically significant in most experiments, and was highly significant in statistical tests of pooled data. Moreover, consistent with earlier studies of diesel and catalytic-converted petrol exhausts, the limited acute tests clearly showed a greater toxicity on these criteria by irradiated than by non-irradiated diesel exhaust. This is conjectured to be due to oxidant-oxygenated compounds (as yet unidentified) with greater cell-damaging properties. Nonirradiated diesel exhaust, however, was clearly toxic to the system(s) responsible for coping with infection by the inhaled streptococcal pathogen, and conferred an increased sensitivity to infection even after very brief exposure periods of 2 to 6 hours.

It is of interest to note that the effect observed with respect to bacterial infection (streptococcal pneumonia) was absent in the diesel or NO_2 experiments in which the viral pathogen (influenza) was used, and was clearly apparent only in one of three experiments testing acrolein (which was very active with bacterial infectivity). From these limited data it is inferred that the difference is probably due to different mechanisms, and their sensitivities, responsible for defense against this viral than against the bacterial pathogen.

The two subacute DE - *Salmonella* experiments were inconclusive with regard to effects on susceptibility to enteric infection. In both experiments mice were challenged after about two weeks exposure to DE, but although mortality rates in test groups were somewhat greater than those in controls, they were not convincingly so (44 vs 41 and 100 vs 80 percent, respectively). One problem was the too high mortality in controls. This sometime occurs as a result of various dose or host factors and it was not feasible to conduct further such tests in this program.

The data in Appendix 4 for tests involving acrolein and NO_2 clearly indicate that exposure to either of these agents results in increased susceptibility to *bacterial* infection, (at least by the *Streptococcus* used here). Acrolein was apparently somewhat more toxic than NO_2. Neither acrolein nor NO_2 conclusively affected defenses against the *viral* pathogen. It would appear that much of the diesel toxicity with respect to streptococcal infectivity could be accounted for by the NO_2 and/or AC constituents, but more precise definition of this is needed.

In all but three experiments one or more other infection lethality parameters were adversely affected by test exposure when compared with controls, as might have been anticipated, vis.: a) Earlier deaths (mortality beginning earlier in exposed than in control groups), b) Lower mean number of days to death among animals dying), and/or, c) Earlier modal day of death. Also not surprising is the fact that in most experiments (all but 4) there were at least some days during post-infection observation on which the relative body weight index (RBWI)* showed a deficit in comparison to that of

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$$\text{RBWI} = \frac{\text{Test group mean body weight on day n/group mean body weight on day 0}}{\text{Control group mean body weight on day n/group mean body weight on day 0}},$$

where day 0 was the day of infectious challenge.

controls. This was considered to be a general indication of infection morbidity reflecting malaise and unwillingness or inability to eat and/or drink. Maximum deficits ranged up to 29%, and deficit days ranged up to 29 out of 30 days observed after challenge (before sacrifice of survivors). Since body weight deficits as well as overt illness occurred in many mice that did not die from infection, this type of toxicity (impaired resistance to infection) could perhaps also be translated to the human situation in terms of decrement in productivity (e.g., absenteeism and lower performance).

Appendix 5 summarizes infectivity test mortality data from earlier catalyst-modified gasoline-engine exhaust and the earlier diesel studies, as well as the current study. In all cases irradiated exhaust was more toxic than nonirradiated exhaust in the context of enhancing susceptibility to bacterial infection. In the diesel studies the fuel consumption has been calculated at about 100 g/mi and contribution to test atmosphere about 1.4 g fuel/m^3. These are considerably less than corresponding data for the catalytic exhaust studies, about 200 g/mi and 5-7 g/m^3. Daily exposures were for 16 h in the "gasoline-catalyst study", 20 h in the "pilot diesel study", and 8 h in the "current diesel study." Exhaust dilution factors were roughly similar in all studies (about 14:1 in the gasoline-catalyst and pilot diesel, and 18:1 in the current diesel). With this background, although the data on excess mortality over controls for the 3 studies are not strictly comparable (tests were run in same manner but not concurrently), a general comparison among these studies (diesels vs gasoline-catalyst) tends to indicate that diesel exhaust may be somewhat more toxicologically potent than gasoline-catalyst exhaust, with respect to effect on resistance to bacterial infection in the acute and subacute situations. Excess mortality in the diesel studies was either roughly equal to or was greater than that in the gasoline-catalyst study, yet the exposure index (hours for acute, hours/day for subacute) in diesel studies was similar to or less than that in the gasoline-catalyst study and the fuel mass contributions to exposure in the diesel studies were only about half or less of those in the gasoline-catalyst study. The chronic test data are equivocal because not only were exposure regimens quite different, but the mortality in control mice in the diesel study was too high for a meaningful comparison.

ACKNOWLEDGMENT

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APPENDIX 1. - DIESEL INFECTIVITY TESTS DESIGN - SUMMARY

MODEL

- Control/test exposure + infectious exposure → infection (Mortality, etc.)

TEST ATMOSPHERES

- Diesel exhaust (non-irradiated, irradiated) - 18:1 dilution
- Acrolein - 4 ppm, 2 ppm
- Nitrogen dioxide - 2.5 ppm

TEST EXPOSURE DURATION ROUTE: INHALATION (WHOLE BODY)

- Acute - 2 hr., 6 hr.
- Subacute - 7, 15, 16 da. (8 hr/da x 7 da/wk)
- Chronic - 44/46 wks (8 hr/da x 7 da/wk

INFECTIOUS MICROBIAL AGENT(S) AND EXPOSURE

- Bacterial
  - Streptococcus pyogenes, Type c B-hemolytic (respiratory pathogen). Respirable aerosol of diluted culture, by inhalation, usually 15 min.
  - Salmonella typhimurium (enteric pathogen). By intragastric intubation of diluted culture.
- Viral - Influenza, A/PR8-34, mouse adapted. Aerosol, by inhalation, 30 min.
- Exposure/dose - Factors adjusted to produce minimal positive response in control subjects (5-20% mortality).

RESPONSE TO INFECTION

- Reflects whether resistance to infection is impaired.
- Mortality - compare test vs. control group mortality.
- Possibly other end points (e.g., time to death, comparative body weight changes).

SUBJECT

- Mouse, female, CR/CD-1, 17-25g (means), 4-6 wks for most.

STATISTICS

- Chi-Square (uncorr.) for mortality comparison.

APPENDIX 2. - MEAN AEROMETRY FOR INFECTIVITY STUDIES
(Average concentrations in chamber atmospheres)

Exp't		CO	HC	NO	NO_2	SO_2	TSP
AB-3	DE	22.2	9.5	15.7	1.8	1.4	7.9
	CA	2.7	4.0	.17	.03	.42	
AB-4	DE	21.3	8.8	13.3	3.6	2.8	5.9
	CA	1.7	2.9	.03	.03	.70	
AB-5	DE	19.6	7.3	11.4	2.0	.91	6.5
	CA	1.2	3.4	.12	.10	.63	
AB-6	DE	19.7	8.0	9.9	2.6	2.3	6.9
	CA	2.7	4.0	.06	.05	.94	
AB-7	DE	19.4	8.9	12.1	1.9	1.2	5.3
	CA	2.1	4.5	.05	.02	.72	
AV-1	DE	21.6	8.7	11.4	1.8	.91	7.2
	CA	2.3	3.8	.17	.13	.67	
AV-2	DE	19.7	8.0	9.9	2.6	2.3	6.9
	CA	2.7	4.0	.06	.05	.94	
AV-3	DE	20.1	8.5	12.2	2.3	1.6	7.0
	CA	2.5	4.7	.08	.02	.73	
SB-2,3	DE	21.0	8.6	13.9	3.2	2.8	6.9
	CA	2.3	3.5	.08	.04	.66	
SB-4	DE	18.6	7.3	9.8	2.5	2.1	7.3
	CA	2.1	3.3	.06	.04	.56	
CB-1,2	DE	19.8	7.4	11.2	2.8	1.9	6.4
RANGE	DE	19-22	7-10	10-16	1.8-3.6	0.9-2.8	5.3-7.9
	CA	1.2-2.7	2.9-4.7	0.03-0.17	0.02-0.13	0.04-0.94	

AB=acute bacterial; AV=acute viral; SB=subacute bacterial; CB=chronic bacterial; DE=diesel exhaust; CA=clean air. All data as ppm by volume except TSP, which are as mg/m^3.

Appendix 3. - MORTALITY IN MICE EXPOSED TO CLEAN AIR OR DIESEL EXHAUST THEN PATHOGEN

Duration of Exposure		Pathogen[a]	EXPOSURE ATMOSPHERE & EFFECT[b]			Statistical Test P value (X^2)		
			Clean Air (CA, Controls)	Diesel Exhaust				
				Non-Irrad. (NI)	Irradiated (I)			
Acute	2 hr	Strept.	5	0	20	0.0059		Pooled: NI v I .039 CA v NI 2 x 10^{-6}
	6 hr		10	20	55	.004 CA v Ex .026 NI v I .022		
			5	15		.29	Pooled .090	
			0	10		.15		
			0	35		.004		
			20	55		.022		
			5	55		5.6 x 10^{-4}		
		Virus	10	10		1.0 NS[c]		
			20	21		.6 NS		
			20	10		... NS		No sig. Effect
Subacute	8 da		30	5		... NS		
		Strept.	11	60		.0018		Pooled: CA v NI=0
	15 da		10	50		.0058		
	16 da		5	50		.0014		
	15 da		20	95		1 x 10^{-6}		
Chronic	44 wk		53	100		.001		Pooled: CA v NI 6 x 10^{-6}
	46 wk		60	100		.002		

[a] Bacterial = Strep. pyogenes; virus = A/PR8-34 influenza
[b] Data are percent mortality, group n = 20
[c] NS = Not significant

APPENDIX 4. - LETHAL RESPONSE TO INFECTION AFTER EXPOSURE TO ACROLEIN OR NITROGEN DIOXIDE

Exposure Duration	Pathogen	Exposure Atmosphere and Effect*				Statistical X^2, p value	
		Clean air (Controls)	Acrolein 2ppm	Acrolein 4ppm	NO_2 2.5ppm		
Acute (6 hr.)	Strept.	0		100		0	Pooled: CA v AC, p~0 CA v NO_2, p=0.0052
		5	95		10	O/A $6x10^{-9}$	
		10			55	~0.001	
	Virus	75		100		<0.02	Not signif.
		20	15		15	>0.5	
Subacute (7 da.)		15			10	>0.5	
		30	15		10	O/A>0.1	
	Strept.	11	60		40	<0.002	CA v AC, p=0.0018 CA v NO_2, p=0.043

n = 20 AC = acrolein O/A = overall
* Data are percent mortality

APPENDIX 5. - INFECTIVITY TEST MORTALITY IN GASOLINE AND DIESEL EXHAUST STUDIES

Exposure	Duration	Infection Mortality			Excess % Mortality[b]		Toxicity Comparison and Statistical Probability[c]	
		CA Controls	Exposed[a]					
			NI	I	NI	I		
Gasoline exhaust, catalytic, 16 h dly (TAME - P,Q)	Acute 6 h	15/100 15	36/100 36	32/60 53	21	38	NI, I>CA $<10^{-5}$ I > NI 0.017	
	Subac 13-16d	24/100 24	39/100 39		15		NI>CA .016	
	Chron 66d	5/20 25	13/20 65		40		NI>CA .013	
Diesel exhaust, 20 h dly	Acute 6 h	11/80 14	36/80 45	44/80 55	31	41	NI, I > CA .0012 I>NI .014	
(Pilot Study, TADE - I)	Subacute 14d	4/39 10	24/39 62	17/39 44	52	34	NI, I > CA <.001	
Diesel exhaust,	Acute 2 h	1/20 5	0/20 0	4/20 20	--	15	O/A 0.059	Pooled:
8 h dly (Main, current study, TADE - II)	6 h	2/20 10	4/20 20	11/20 55	10	45	O/A .004 NI, I > CA .026 I > NI .022	O/A 6 x 10^{-4} NI,I > CA 0.030 NI vs I 0.039
	6 h	9/120 7.5	38/120 31.7		24.2		NI > CA 2 x 10^{-6}	
	Subacute 8,15d	9/78 11.5	51/80 63.8		52.2		NI > CA ~ 0	
	Chron 44,46wk	22/39 56.4	36/36 100		43.6		NI > CA 6 x 10^{-6}	

CA = clean air; O/A = overall

[a] NI = nonirradiated; I = irradiated

[b] Compared to controls

[c] Chi square test (uncorrected)

Session V

MUTAGENIC AND CARCINOGENIC POTENCY OF EXTRACTS OF DIESEL AND RELATED ENVIRONMENTAL EMISSIONS

Chairman:

Dr. Stephen Nesnow

Mutagenic and Carcinogenic Potency of Extracts of Diesel and Related Environmental Emissions: Study Design, Sample Generation, Collection, and Preparation.
Huisingh, J. L., R. L. Bradow, R. H. Jungers, B. D. Harris, R. B. Zweidinger, K. M. Cushing, B. E. Gill, and R. E. Albert.

Mutagenic and Carcinogenic Potency of Diesel and Related Environmental Emissions: Salmonella Bioassay.
Claxton, Larry D.

Mutagenic and Carcinogenic Potency of Extracts of Diesel and Related Environmental Emissions: In Vitro Mutagenesis and DNA Damage.
Mitchell, Ann D., Elizabeth L. Evans, Mary Margaret Jotz, Edward S. Riccio, Kristien E. Mortelmans, and Vincent F. Simmon.

Mutagenic and Carcinogenic Potency of Extracts of Diesel and Related Environmental Emissions: In Vitro Mutagenesis and Oncogenic Transformation.
Casto, Bruce C., George G. Hatch, Shiu L. Huang, Joellen L. Huisingh, Stephen Nesnow, and Michael D. Waters.

Session V

(Continued)

Mutagenic and Carcinogenic Potency of Extracts from Diesel Related Environmental Emissions: Simultaneous Morphological Transformation and Mutagenesis in BALB/c 3T3 Cells.
Curren, R. D., R. E. Kouri, C. M. Kim, and L. M. Schechtman.

Mutagenic and Carcinogenic Potency of Extracts of Diesel and Related Environmental Emissions: Two-Stage Carcinogenesis in Skin Tumor Sensitive Mice (SENCAR).
Slaga, T. J., L. L. Triplett, and Stephen Nesnow.

Mutagenic and Carcinogenic Potency of Extracts of Diesel and Related Environmental Emissions: Summary and Discussion of the Results.
Nesnow, Stephen and Joellen L. Huisingh.

MUTAGENIC AND CARCINOGENIC POTENCY OF EXTRACTS OF DIESEL AND RELATED ENVIRONMENTAL EMISSIONS: STUDY DESIGN, SAMPLE GENERATION, COLLECTION, AND PREPARATION

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ABSTRACT

A major diesel emissions research program has been initiated by the U.S. Environmental Protection Agency to assess the human health risk associated with increased use of diesel automobiles. This program is intended to establish the mutagenic and carcinogenic potency of complex organics associated with diesel particles as well as comparative particle-bound organics from other environmental emissions for which human epidemiological data are available.

The mobile source samples selected for this study were collected from a heavy-duty diesel engine, a series of light-duty diesel passenger cars, and a gasoline catalyst automobile. The comparative source samples incorporated into the study were cigarette smoke condensate, coke oven emissions, roofing tar emissions, and benzo(a)pyrene. The samples were tested using three mutagenic assays and four carcinogenic assays as prescribed by a test matrix.

This report describes the study design, particle generation, and sample collection and preparation. A brief summary of the bioassays is also included.

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INTRODUCTION

The complex mixture of incomplete combustion organics associated with diesel particle emissions has been shown to be mutagenic in short-term bioassays (1). These organics contain known carcinogenic polycyclic aromatic hydrocarbons, as well as highly mutagenic polar neutral (oxygenated polycyclic) compounds. A variety of combustion emission products have been recognized as human carcinogens, including chimney soot (2) and coke oven emissions (3). At present, there is no direct evidence that diesel emissions are human carcinogens or that human exposure to environmental concentrations of diesel emissions will increase the incidence of cancer in exposed populations. The expected increase in the use of diesel-powered automobiles and resulting increased ambient air concentrations of diesel emissions have stimulated a major diesel emissions research program being conducted by EPA's Office of Research and Development (4). The information generated from this research will be used to assess the human health risk associated with increased use of the diesel engine.

Diesel exhaust is a mixture of particles (carbonaceous soot) and gases. The principal gases (hydrocarbons, nitrogen oxides, sulfur oxides, and carbon monoxide) are similar to those currently emitted by gasoline engines and other combustion sources. The particles emitted from diesel engines differ significantly in both quantity and composition from gasoline particle emissions. Diesel cars emit 30-to-100 times more particulate matter than gasoline catalyst cars. Gasoline particulate emissions are primarily sulfur compounds, while diesel particles are composed of carbonaceous soot with a large amount (5 - 50%) of complex organics adsorbed to the surface. This study was designed to determine the mutagenic and carcinogenic potency of these complex organics compared to particle-bound organics from other environmental emissions. This report describes the study design, particle generation, and sample collection and preparation for biological studies. The results and a summary of the relative mutagenic and carcinogenic potency of these organics are included elsewhere in this volume.

STUDY DESIGN

The objective of this study was to determine the relative mutagenic and carcinogenic potency of the extractable organics from diesel emissions and other emissions for which human epidemiological data are available.

Epidemiological studies have shown that a dose-response relationship exists between exposure to coke oven emissions and human lung cancer rates (5, 6, 7, 8). Increased incidence of cancer has also been shown in epidemiological studies of humans exposed to roofing tar emissions (9) and cigarette smoke (10). Environmental exposure to various hydrocarbon combustion products, measured by the benzo(a)-pyrene (BaP) concentration, has also shown a highly consistent carcinogenic potency in different epidemiological studies for coke oven, roofing tar, and gas works emissions (11). Coke oven emissions, roofing tar emissions, and benzo(a)pyrene were, therefore, selected as comparative sources for this study. Cigarette smoke condensate (CSC) was selected as an additional comparative sample because of the large number of animal studies that have been conducted with this source (12).

The mobile source samples selected for this study included a heavy-duty diesel engine (Caterpillar 3304), a series of light-duty diesel passenger cars (Datsun Nissan 220C, Oldsmobile 350, and Volkswagen turbocharged Rabbit), and a gasoline catalyst car (Fort Mustang II).

The design of the sample and bioassay test matrix is shown in Table 1. The mutagenesis bioassays, selected to detect gene mutations, chromosomal effects and DNA damage, were all short-term *in vitro* assays performed in microbial or mammalian cells. The carcinogenesis bioassays included both

TABLE 1. TEST MATRIX FOR DIESEL AND COMPARATIVE SOURCE STUDY

Test Procedure	Mobile sources								
	Diesel				Gasoline	Comparative Sources			
	CAT	NISSAN	OLDS	VWRAB	MUSTANG	CSC	Coke	Roof tar	BaP
Mutagenesis									
Gene mutations	X	X	X	X	X	X	X	X	X
Chromosomal aberration		X	X			X	X	X	X
DNA damage	X	X	X	X	X	X	X	X	X
Carcinogenesis									
Oncogenic transformation	X	X	X	X	X	X	X	X	X
Skin carcinogenesis	X	X	X	X	X	X	X	X	X
Pulmonary adenoma model			X			X	X		X
Intratrachael instillation			X			X	X		X
Human Epidemiology									
Available						X	X	X	

in vitro mammalian cell assays and *in vivo* bioassays. The *in vivo* assays included skin carcinogenesis (Senecar and C57 black mice), pulmonary adenoma formation (strain A mice), and intratracheal instillation (hamsters).

SAMPLE GENERATION AND COLLECTION

The mobile and comparative source samples were provided for this study by the organizations shown in Table 2. Sample collection techniques are summarized in Table 3.

TABLE 2. SAMPLE SUPPLIERS*

Sample source		EPA	Other
Diesel	CAT	R. Bradow/ESRL	-
	NISSAN	R. Bradow/ESRL	-
	OLDS	R. Bradow/ESRL	-
	VW RAB	R. Bradow/ESRL	J. Sturm/DOT
Gasoline	MUSTANG	R. Bradow/ESRL	-
Comparative sources	CSC	J. Smith/HERL	W. Griest/ORNL
	Coke	B. Harris/IERL	K. Cushing/SRI
	Roof tar	B. Harris/IERL	-

*ESRL = Environmental Sciences Research Laboratory, DOT = Department of Transportation, HERL = Health Effects Research Laboratory, ORNL = Oak Ridge National Laboratory, IERL = Industrial Environmental Research Laboratory, SRI = Southern Research Institute

TABLE 3. SAMPLE COLLECTION TECHNIQUES

Sample source		Sampling apparatus	Media
Diesel	CAT	Dilution tunnel	Pallflex T filter
	NISSAN	Dilution tunnel	Pallflex T filter
	OLDS	Dilution tunnel	Pallflex T filter
	VW RAB	Dilution tunnel	Pallflex T filter
Gasoline	MUSTANG	Dilution tunnel	Pallflex T filter
Comparative sources	CSC	Refrigerated condenser	Acetone
	Coke	Massive Air Volume	ESP plates
	Roof tar	Baghouse	Teflon filter bags

DIESEL AND GASOLINE

The mobile source particulate emission samples used in this study were generated from the sources shown in Table 4. The diesel engine and vehicles were all operated on the same lot of No. 2 diesel fuel. The gasoline vehicle was operated on unleaded gasoline at a richer-than-normal stoichiometry in order to produce sufficient sample volume for limited studies. The particle emission rates are shown in Table 5.

TABLE 4. MOBILE SOURCE SAMPLE DESCRIPTIONS

Sample source		Description	Fuel	Driving cycle
Diesel	CAT	Caterpillar 3304	Diesel No. 2	Mode II
	NISSAN	Nissan Datsun 220C	Diesel No. 2	HWFET
	OLDS	Oldsmobile 350	Diesel No. 2	HWFET
	VW RAB	Volkswagen turbocharged Rabbit	Diesel No. 2	HWFET
Gasoline	MUSTANG	1978 Mustang II-302*	Unleaded gasoline	HWFET

*V-8, equipped with catalytic converter and exhaust gas recirculation (EGR)

TABLE 5. PARTICLE EMISSION RATES FOR MOBILE SOURCES

Sample source		Emission rate g/hp/h	 g/mi
Diesel	CAT	0.72	
	NISSAN		0.33
	OLDS		0.52
	VW RAB		0.18
Gasoline	MUSTANG		0.0053

To simulate actual driving patterns, the vehicles (except for the Caterpillar) were operated on a chassis dynamometer, using the highway fuel economy test cycle (HWFET): average speed of 48 miles per hour in 12.75 minutes over 10.24 miles. The Caterpillar engine was mounted on an engine dynamometer and operated at Mode II using a steady-state operation of 2200 rpm and an 85-pound load. All of the mobile source particulate emission samples, except those from the VW Rabbit, were generated in the chassis dynamometer and engine dynamometer facilities of EPA's Environmental Sciences Research Laboratory in Research Triangle Park, North Carolina. The VW Rabbit samples were provided through an interagency agreement with the Department of Transportation.

Particulate samples were collected using dilution tunnel sampling technique (13). The emissions were diluted to obtain realistic samples of particle-bound organics. Because of the high temperatures (>200° C) in the tailpipe of an operating diesel engine, organic materials are generally in the gas phase; thus, soot filtered at these temperatures would contain very little extractable organic material. In the ambient air, however, particle and gaseous exhaust is quickly cooled and diluted; as the overall temperature is reduced, carbon particles begin to adsorb organic material. The dilution tunnel technique simulates this process.

The heavy-duty Caterpillar engine exhaust was split before dilution in the tunnel to ensure that exhaust volumes did not exceed the capacity of the dilution tunnel system. The total exhaust from the passenger cars was diluted approximately 10-fold with filtered air prior to the sampling. After dilution, the particulate matter was collected by filtration in large-scale fiber samplers, using Teflon-coated glass Pallflex T 68-20 filters at a flow rate of 100 cfm.

CIGARETTE SMOKE CONDENSATE

The CSC was generated and collected by the Tobacco Smoke Chemistry Group of the Oak Ridge National Laboratory (ORNL) Analytical Chemistry Division for the Chemical Repository Program. It should be noted that humans are actually exposed to smoke particulate matter, not condensate, and that there may be significant chemical differences between the two. The cigarettes chosen for making the CSC were the Kentucky Reference 2R1, a remake of the widely referenced 1R1. The cigarettes are 85-mm (king size), non-filter, typical of U. S. cigarettes consumed from 1962 - 1966.

The CSC generator loads, lights, smokes, and ejects 2,000 cigarettes per hour. A maximum of 10 puffs per cigarette is taken at a rate of 1 puff (2 sec, 35 cc) per minute. As the cigarettes, which are in a large rotating turret, pass smoking ports, a vacuum draws a puff from each of 40 cigarettes in different stages of consumption. The smoke is mixed and drawn through traps, which are partially filled with acetone and immersed in a refrigerated, dry ice-isopropanol bath.

For this study, approximately 50,000 cigarettes were consumed to produce approximately 1 kg of CSC. The CSC was removed by rinsing the trapping system with a minimal amount of acetone.

COKE OVEN

The coke oven emissions were collected by sampling on top of a coke oven battery at Republic Steel in Gadsden, Alabama, about 60 miles northeast of Birmingham. Two Massive Air Volume Samplers (MAVS) were positioned side-by-side on top of the north end of the coke oven battery. Due to local wind conditions, various types of aerosols were sampled. The prevailing wind direction was north and north-northwest — from the direction of Gasden; thus, as unknown, but significant, portion of the emissions sampled may have been from the urban environment.

Initially, the electrostatic precipitator (ESP) plates coated with coke oven ambient emissions were rinsed in methylene chloride. Later, however, the impactor and ESP plates were dry-scraped. A relatively small amount of material (about 200 g) was recovered in more than 2100 hours of sampling, indicating that the prevailing wind direction decreased the concentration of suspended dust at the site.

Since the amount of ambient coke oven sample obtained was not adequate to complete all of the in vivo bioassays, an additional large sample of coke oven mains was obtained. The collection and bioassay of this sample will be reported later.

ROOFING TAR

The roofing tar emissions were generated and collected by the Process Measurement Branch of EPA's Industrial Environment Research Laboratory in Research Triangle Park. The North Carolina Department of Transportation provided the tar pot and the pitch-based tar for these studies. The tar pot was a conventional unit with an external burner. A 6-foot stack extension was added to the test unit to prevent the burner from entering the sampling hood, which was mounted over the tar pot. A Teflon-coated aluminum pipe led from the hood to a small baghouse. Special nonreactive Teflon filter bags were fitted in the baghouse to collect the particulate emissions.

The sampling was conducted for 8 hours, during which the tar temperature was held between 360° - 380° F to maintain the tar in liquid form. Particulate matter was collected from the Teflon filter bags, and by scraping the collector duct and pot bottom.

SAMPLE PREPARATION

The mobile source and comparative emission samples, except the CSC, were soxhlet-extracted with dichloromethane (DCM) to remove the organics. DCM was found to be the most effective of a series of solvents in removing mutagenically active organics from diesel particles in preliminary studies using the Salmonella typhimurium mutagenesis bioassay.

Soxhlet-extraction of the mobile sources particle-laden filters was accomplished by placing 12 - 16 filters, without thimbles, in a 2.3 liter side-chamber extractor for 48 hours. The Teflon filter bags containing roofing tar emissions were cut into sections and soxhlet-extracted for 72 hours (until clear). The coke oven emissions scraped from the ESP plates were soxhlet-extracted in thimbles in a standard size extractor for 24 hours.

The soxhlet-extracted organics were filtered using Teflon millipore filters (0.2 μm) to remove particles. Large volumes of solvent (mobile source samples) were concentrated to a smaller volume by rotary evaporation under reduced pressure. Aliquots were evaporated to dryness under nitrogen and stored frozen in the dark. The percent extractable and benzo(a)pyrene analysis results are given in Table 6.

TABLE 6. RESULTS OF EXTRACTION AND BENZO(A)PYRENE ANALYSIS

Sample source		Extractable matter percent	Benzo(a)pyrene	
			$\frac{\text{ng BaP}}{\text{mg extract}}$	$\frac{\text{ng BaP}}{\text{mg particulate}}$
Diesel	CAT	26-27	2	0.5
	NISSAN	4-8	1173	96.2
	OLDS	12-17	2	0.4
	VW RAB	18	26	4.6
Gasoline	MUSTANG	39-43	103	44.1
Comparative sources	CSC	-	<1	-
	Coke	5-10	478	31.5
	Root tar	>99	889	889

The CSC, after being drawn through the refrigerated condenser, was removed from the condenser by rinsing with a minimal amount of acetone. The acetone suspension was then placed on a rotary evaporator and, at reduced pressure and 30° C, the semi-volatiles were removed; evaporation was continued until a constant weight was achieved. The tar content of the CSC-acetone suspension was adjusted to require levels by the addition of appropriate amounts of acetone. The acetone was removed from the necessary samples by evaporation in a vacuum desiccator over Drierite. This treatment removed the acetone solvent, but most of the CSC water remained with the sample.

The samples prepared as described above, were furnished to the Health Effects Research Laboratory in Research Triangle Park, North Carolina, (HERL-RTP) by the suppliers noted earlier. At HERL-RTP, the samples were coded, using a distinguishing number for each source and then assigning random numbers to each sample, creating a blind experiment. The coded samples were then aliquoted according to the bioassay requirements.

All shipments were via air express, on dry ice, according to the transportation procedures for potentially hazardous biologicals/chemicals specified by the Department of Transportation. To ensure stability, the samples were stored in the dark at or below -30° C and bioassayed soon after receipt.

Prior to conducting the bioassay(s), the investigator(s) added dimethyl sulfoxide (DMSO) to the samples to achieve the desired concentration (mg sample/ml). The same DMSO solvent was used as a control. For some specified assays (i.e., skin carcinogenesis and oncogenic transformation), acetone was used as the solvent and the control.

BIOASSAYS

The mutagenesis bioassays were selected to detect gene mutations, chromosomal effects, and DNA damage. The results of the mutagenesis test matrix reported in this volume are for the following bioassays:

- reverse mutation in *Salmonella typhimurium*
- forward mutation in L5178Y mouse lymphoma cells
- forward mutation in Balb/c 3T3 mouse embryo fibroblasts
- forward mutation in Chinese hamster ovary cells
- mitotic recombination in *Saccharomyces cerevisiae*
- DNA breakage in Syrian hamster embryo cells
- sister chromatid exchange in Chinese hamster ovary cells.

The carcinogenesis bioassays were selected to provide data as early as possible from in vitro and short-term in vivo assays (e.g., skin tumor initiation). The longer term in vivo assays (e.g., complete skin carcinogenesis and intratracheal instillation) will allow scoring and pathological examination for carcinomas. The results of the carcinogenic testing, completed to date and reported in this volume, including the following bioassays:

- oncogenic transformation in Balb/c 3T3 cells
- viral enhancement of transformation in Syrian hamster embryo cells
- skin tumor initiation in Sencar mice.

The bioassays were performed, if possible, in such a way that dose-response data would demonstrate a positive increase (slope) above spontaneous levels for at least three doses. This usually requires a preliminary toxicity test over a wide dose range followed by a test conducted at 5-to-7 doses spaced over half-logs. Where such dose-response data were obtained, the initial linear slope of the response curve was determined and used to provide comparative potency rankings of the samples. In several assays (e.g., the Balb/c 3T3 cells), dose-response data were not obtained and the lowest effective (positive) dose tested was determined. Difficulties in obtaining good dose-response data may be due, in part, to difficulties in delivering increasing doses of the sample to the cells due to solubility problems.

The short-term bioassays generally included positive and negative (solvent) controls. The assays were repeated and, in some cases, run in more than one laboratory. Samples were decoded after submission of the final results. The papers which follow describe the results determined by each bioassay laboratory. The final paper of this session summarizes all of the results to date (14).

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MUTAGENIC AND CARCINOGENIC POTENCY OF DIESEL AND RELATED ENVIRONMENTAL EMISSIONS: SALMONELLA BIOASSAY

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ABSTRACT

Due to the expected increase in the percentage of diesel vehicles in the United States, the Environmental Protection Agency must evaluate the health effects associated with exposure to diesel emissions. Respirable particles from a variety of combustion sources have the potential of being carcinogenic and mutagenic. The objective of these studies was to determine the relative biological activity of the organic material adsorbed on these particles in *in vitro* mutagenesis bioassays. The organic extracts from the following series of emission sources were quantitative bioassayed in the *Salmonella* assay for mutagenic activity: (1) a light-duty Oldsmobile diesel 350 engine; (2) a heavy-duty Caterpillar diesel engine; (3) a light-duty Nissan engine; (4) a Volkswagen Rabbit diesel engine; (5) cigarette smoke; (6) roofing tar; (7) coke oven; and (8) a gasoline catalyst Mustang. This paper provides a comparison of these sources within the Salmonella bioassay and also demonstrates how bacterial systems can be used as a quality assurance measure in *in vivo* testing.

INTRODUCTION

Bacterial systems have recently been used to demonstrate that organic mixtures from combustion processes, including diesel engines, are mutagenic (1,2). This study demonstrates a means of comparing the mutagenic potential of various sources with microbial test methods. Included within the problem of source to source comparison are variables within: (1) collection methods, (2) methods of chemical extraction, (3) proportion of mutagenic components within the mixture, (4) the amount of "masking" organics that are bactericidal, (5) the metabolizing ability of the indicator organism and exogenous activation systems, and (6) the sensitivity of the bioassay. Although these variables do exist, they can be controlled to some extent. In view of these considerations, this paper demonstrates the utility of comparing organics from various combustion sources. This study also demonstrates how bacterial systems can aid in the identification of active components and can be used as a quality assurance measure in *in vivo* testing.

MATERIALS AND METHODS

The assay system used in this study was the *Salmonella typhimurium* plate incorporation test as developed by Ames *et al*. (3). Four mutant strains of *Salmonella typhimurium* (TA98, T 100, TA1535, and TA98-FR1) were used in this study. The nitroreductase deficient strain TA98-FR1 was obtained from Dr. Herbert Rosenkranz at the New York Medical College, Valhalla, N.Y., and the other strains were obtained from Dr. Bruce Ames at the University of California, Berkeley. All samples were tested in TA98, TA1535, and TA100. The diesel and cigarette smoke samples were assayed also with TA98-FR1.

The protocol described by Ames *et al*. (3) was employed with minor modifications described below. All samples were tested with and without Aroclor-induced S-9 as described by Ames (3). Two minor modifications to the protocol were used. First, the minimal level of histidine was added to the plate media rather than to the overlay. Secondly, plates were incubated for 72 hours rather than 48 hours. Each plate was counted electronically using an Artek Model 880 automatic colony counter. The positive controls used for TA98 and TA98-FR1 were 2-nitrofluorene for without activation and 2-anthramine for the S-9 activation control. For TA100 and TA1535, sodium azide was substituted for the positive control without activation. All quality assurance procedures as described by Ames (3) and the Washington Committee on the Ames Test (4) were followed. In addition, all experiments were conducted in a laminar flow biological exhaust hood under yellow lights. When sufficient sample was present, each extract was tested in triplicate at a minimum of five doses.

The linear portion of the dose response curve for samples positive within the test were used to calculate a linear regression line. The equation of that line was used to calculate the expected response at 100 μg of organic material (a dose located within the linear response range). This value was termed the specific activity and was simply a convenient method of comparing the responses of different samples. The specific activity values for each sample are found in Table 1. In keeping with companion papers (5), the activity of each sample was compared to the activity of the Nissan sample by arbitrarily assigning the Nissan sample a relative value of 100. These comparisons are referred to as Relative Potency and are found in Table 2.

TABLE 1. SPECIFIC ACTIVITIES AT 100 μg OF ORGANIC MATERIAL

Sample	TA98		TA100	
	+S9	-S9	+S9	-S9
		Diesel		
Caterpillar	59.3	65.9	115.2	167.8
Nissan	1367.1	1225.2	881.7	1270.1
Oldsmobile	318.7	614.8	169.9	247.5
VW	297.5	399.2	426.0	641.6
		Gasoline		
Mustang	341.9	137.8	228.0	196.5
		Comparative Samples		
Cigarette	98.2	Neg	?	Neg
Coke Oven	251.6	164.1	265.6	259.4
Roofing Tar	98.7	Neg	420.0	Neg
		Control Compound		
B(a)P	15202.3*	NT	26438.0*	NT

*Extrapolation.
NT = Not tested.
Neg = Negative.

TABLE 2. RELATIVE POTENCY OF ORGANIC MATERIAL

Sample	TA98 +S9	TA98 -S9	TA100 +S9	TA100 -S9
		Diesel		
Caterpillar	4.3	5.4	13.0	13.2
Nissan	100.0	100.0	100.0	100.0
Oldsmobile	22.3	50.2	19.3	19.5
VW	21.8	32.6	48.3	50.6
		Gasoline		
Mustang	25.0	11.3	25.9	15.5
		Comparative Samples		
Cigarette	7.2	Neg	?	Neg
Coke Oven	18.4	13.4	30.2	20.4
Roofing Tar	7.2	Neg	47.6	Neg
		Control Compound		
B(a)P	1112.1*	NT	2997.5*	NT

*Extrapolation.
NT = Not tested.
Neg = Negative.

The description of the sample collection procedures, extraction, and solvent exchange to DMSO is found in a companion paper (6).

TABLE 3. AVERAGE NUMBER OF REVERTANTS PER PLATE AT 100 μg OF ORGANIC WITH OLDSMOBILE AND CATEPILLAR QUALITY CONTROL SAMPLES

Month	Sample:	Olds	Cat	Olds	Cat
	AC7:	-S9	-S9	+S9	+S9
January		633.33	170.67	318.33	115.00
February		591.00	(131.80)*	393.33	(173.20)*
March		628.33	168.67	362.50	122.00
April		N.T.	N.T.	N.T.	N.T.
May		605.33	203.33	305.00	135.67
June		739.33	218.00	421.33	137.00
July		746.67	200.33	366.00	126.00
August		701.00	169.33	422.33	126.33

*Calculated

RESULTS

This study was divided into three areas: (1) the comparison of various combustion sources using microbial mutagenesis, (2) the monthly bioassaying of sample aliquots as a quality assurance for concurrent mouse skin painting studies (7), and (3) the use of a nitroreductase deficient strain to determine if a diesel extract contained active nitrogenated compounds.

When a mammalian activation system was added, each combustion source sample was shown to have mutagenic activity (Tables 1 and 2). Cigarette smoke condensate, roofing tar, and benzo(a) pyrene required metabolic activation in order to obtain a positive response. The other samples contained direct acting mutagens. The majority of the activity associated with the diesel samples was direct acting. The gasoline automobile sample had direct-acting mutagens; however, the addition of a mammalian activation system increased the activity of this sample. All samples were negative with strain TA1535.

Two samples illustrate the use of microbial bioassays for quality assurance. The Caterpillar sample and Oldsmobile sample were tested monthly in order to determine if the mutagenic activity of organic extracts was altered with prolonged storage. Table 3 shows the results for the monthly Caterpillar and Oldsmobile samples tested to date at 100 μg of organics per plate. Each graph point represents the average of three plates. No significance variation was found in the mo[illegible] to-month test results.

Only two sampl[illegible] the diesel Nissan and cigarette smoke condensate samples, provided sufficient material for testing with the nitroreductase strains. TA98 and TA98-FR1 gave very similar results with the cigarette smoke condensate; however, TA98-FR1 gave one-half the response of TA98 with the diesel sample.

DISCUSSION

Each organic sample from the various combustion source emissions was demonstrated to be mutagenic in the *Salmonella typhimurium* mutagenesis assay. This study demonstrates that emission organics can be compared within bacterial assay systems and that microbial assays can supply important supplemental information.

The bacterial assay demonstrated that the mutagenic substances associated with organic emissions were different for each type of emission. For example, the cigarette smoke condensate required activation before mutagenic activity was seen; however, most of the activity associated with the diesel samples was direct acting. Secondly, the range of activity found with the diesel sample spanned the range of activities associated with the comparative samples. Next, the lack of response in strain TA1535 suggests that most, if not all, of the activity is due to polynuclear frameshift mutagens and not to alkylating agents. The nitroreductase deficient strain demonstrated a difference between the two samples available for testing. The responses of TA98 and TA98-FR1 were markedly different with the diesel Nissan sample but quite similar with the cigarette smoke condensate. This would suggest that the particle bound organics from the Nissan diesel contains inactive nitro compounds that are reduced to an active form by nitroreductase enzymes which are found in TA98.

The calculation of relative potencies is a useful means of understanding the range activity available from various combustion organics. As can be easily seen from Table 2, the maximum attainable difference within this study was seen between the Caterpillar and Nissan samples which demonstrated approximately 25-fold difference. The quality assurance portion of this study provides evidence that the active components in the microbial mutagenesis assay are stable when stored as the extract at -70°C for at least seven months.

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General Discussion

J. SIAK: Do you have any data on the Oldsmobile or the Volkswagen on the nitro-reductant strains?

L. CLAXTON: No, we did not have enough samples at that particular time to go back and do those. We were just lucky to have a little bit of sample left of those two and we were hoping to go back with a variety of samples and use this strain on them.

J. SIAK: You didn't show any data on characterization of fractions from automobiles. Are you going to do some of those studies?

J. HUISINGH: I'm sorry that it was not possible to have all the characterization data completed before the bioassay data, but we were pushing so hard to get all the samples generated and all the bioassays performed. Unfortunately, the fractionation data had to be done under contract which took some time to be negotiated so that those fractions are just now being prepared. So it will be a little time before we have all the chemical characterizations data on these samples. I should say something that I failed to say in my presentation. The coke oven that was selected by Republic Steel for us to put that massive volume sampler near was a clean coke oven and the location was oftentimes in the opposite direction of the wind blowing, which almost always blows.We collected that coke oven sample, since it was an ambient sample, mixed with gas and air, and we had hoped maybe in six months of sampling, an adequate sample could be collected for the whole battery of testing including the intratracheal installation. That just was not possible and it looked like we would be there for years collecting this sample. So they had to go back and get a coke oven sample from the main of the coke oven which is not quite the same as an ambient emission sample. That sample has now arrived and will be put through this battery of tests, and it is that sample that will be used in the intratracheal inhalation. Later we will report on the comparison between the coke oven mains and the ambient sample.

D. CHOUDHURY: Was your coke oven sample kept in solution or as a dry extract?

L. CLAXTON: It was kept as a dry extract and was stored at minus 80 degrees centrigrade in a Revco freezer.

D. CHOUDHURY: So every time it was tested, it was a fresh solution?

L. CLAXTON: Yes.

B. BOWARD: Did I understand correctly that each of the diesel engines you referred to, except for the caterpillar were in transient. These were chassied down on a test except for the caterpillar?

L. CLAXTON: Yes.

D. HOFFMANN: I saw your tobacco tar data and compared it with other laboratories. Since the Ames Test is not 100 percent reproducible, did other laboratories test your samples?

L. CLAXTON: Yes, we have done this on quite a few diesel samples. We have an on-site contractor at my laboratory, Stanford Research Institute, which have run all very similar samples, and in some cases the same sample, and they are consistent with each other.

T. BAINES: When you take your data at the 100 microgram point, does this not include then the spontaneous portion of the curve?

L. CLAXTON: As we are calculating it, yes, it does.

T. BAINES: How does that influence one's ability to make or to take the mass emissions and incorporate them into the results to get comparative data?

L. CLAXTON: I think you would have to adjust this data differently if you wanted to take the mass emissions and start to do revertents per mile or any such thing as that.

T. BAINES: How would you recommend doing that?

L. CLAXTON: You could calculate this. I think what you are asking is that since we are including the spontaneous revertants would not spontaneous influence our total result in such a way as to make them not comparable. To some extent it would, but I think you could simply do a net revertents and then do your slope which would actually be the same. It is not going to affect results greatly and you would have to look at each strain individually becuase the responses by strain are somewhat different.

T. BAINES: Your caterpillar showed remarkably low bio-activity. Do you know of any reason why that might have been quite low especially in light of the fact that I think the other caterpillar, the 3208, was significantly higher in this activity?

L. CLAXTON: Your guess is as good as mine.

MUTAGENIC AND CARCINOGENIC POTENCY OF EXTRACTS OF DIESEL AND RELATED ENVIRONMENTAL EMISSIONS: IN VITRO MUTAGENESIS AND DNA DAMAGE

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ABSTRACT

The *Saccharomyces cerevesiae* D3 recombinogenic assay, the assay for forward mutagenesis in L5178Y mouse lymphoma cells, and the sister chromatid exchange (SCE) assay using Chinese hamster ovary cells were used to evaluate the *in vitro* mutagenic and DNA-damaging effects of eight samples of diesel engine emissions and related environmental emissions. The recombinogenic assay was not sufficiently sensitive for this evaluation, but mutagenicity was detected in the L5178Y mutagenesis assay following exposures of the cells to all of the emission samples, and DNA damage in the SCE assay was induced by most of the emission samples in the presence and absence of metabolic activation. The observation of positive results in the absence of activation indicated that the samples contained substances that were direct-acting mutagens and DNA-damaging agents.

INTRODUCTION

The potential of samples of diesel engine emissions and related environmental emissions to induce *in vitro* mutagenicity and DNA damage were evaluated by three approaches: the mitotic recombinogenic assay utilizing the yeast *Saccharomyces cerevisiae* D3; the assay for forward mutagenesis at the thymidine kinase locus in L5178Y mouse lymphoma cells; and the sister chromatid exchange (SCE) assay using Chinese hamster ovary cells.

All testing was performed in the absence and in the presence of an Aroclor 1254-stimulated metabolic activation system since some carcinogenic chemicals (e.g., of the aromatic amino type or the polycyclic hydrocarbon type) may be inactive unless they are metabolized to active forms. In animals and man, an enzyme system in the liver or other organs (e.g., lung or kidney) is capable of metabolizing a large number of these chemicals to carcinogens, and some of these intermediate metabolites are very potent mutagens and DNA-damaging agents.

The testing was conducted using coded samples. Upon decoding, the samples were identified as three mixtures having known carcinogenicity--coke oven emission, roofing tar emission, and cigarette smoke condensate; the emission of a gasoline (Mustang) engine; an emission from a heavy-duty diesel (Caterpillar) engine; and emissions from three diesel automobile engines, identified as Nissan, VW rabbit, and Oldsmobile.

Saccharomyces cerevisiae D3 Recombinogenic Assay

The yeast *S. cerevisiae* D3 is a diploid microorganism heterozygous for a mutation leading to a defective enzyme in the adenine-metabolizing pathway (1). When grown on medium containing adenine, cells homozygous for this mutation produce a red pigment. These homozygous mutants can be generated from the heterozygotes by mitotic recombination. The frequency of this recombinational event may be increased by incubating the organisms with various carcinogenic or recombinogenic agents. The recombinogenic activity of a compound or its metabolite is determined from the number of red-pigmented colonies appearing on test plates (2).

L5178Y Mouse Lymphoma Mutagenesis Assay

The L5178Y mouse lymphoma assay (3-5) measures the effects of chemicals on the forward mutation frequency of the cells at the thymidine kinase (TK) locus. Heterozygous cells ($TK^{+/-}$) are capable of utilizing exogenous thymidine. However, when the cells are mutated to a homozygous recessive ($TK^{-/-}$) condition, they utilize neither thymidine nor thymidine analogs, the latter of which could otherwise kill the cells competent in TK activity. One effective analog of thymidine is trifluorothymidine (TFT) (6), and only mutated cells can grow and form colonies in the presence of medium supplemented with TFT. Hence, the mutagenic activity of a chemical can be determined by the number of colonies found growing in the presence of TFT.

Sister Chromatid Exchange Assay

The induction of DNA lesions by chemical mutagens leads to the formation of sister chromatid exchanges (SCEs), which may be related to recombinational or postreplicative repair of DNA damage (7-9). Exchanges between the two chromatids of each chromosome are observed in metaphase cells that have been grown in the presence of bromodeoxyuridine (BrdU) for two rounds of replication. Because DNA replication is semiconservative, these chromosomes consist of one chromatid in which both DNA strands are BrdU-substituted and one chromatid in which BrdU substitution is limited to a single strand. These chromatids are stained differentially by the fluorescence-plus-Giemsa (FPG) technique (10), which permits visualization of SCEs by the "harlequin" pattern of darkly and lightly stained chromatid segments produced. The SCE technique is highly sensitive; its use permits detection of the effects of direct-acting chemical mutagens (8,9,11) as well as those that require metabolic activation (12,13) on mammalian cell chromosomes. Wolff (14) and Hollstein et al. (15) have reviewed the chemicals that have been shown to increase SCE frequencies.

MATERIALS AND METHODS

Aroclor 1254-Stimulated Metabolic Activation System

Ames et al. (16) have described the Aroclor 1254-induced liver metabolic activation system that we used for the recombinogenic assays. This reaction mixture consisted of, for 10 ml: 1 ml of S-9 [1 g (wet weight) of liver to 3 ml of 0.15 M KCl buffer]; 0.2 ml of 0.4 M MgCl and 1.65 M KCl; 0.05 ml of 1 M glucose-6-phosphate; 0.4 ml of 0.1 M NADP; 5 ml of 0.2 M sodium phosphate buffer (pH 7.4); and 3.35 ml of H_2O. Similar reaction mixtures were used for the L5178Y mutagenesis and SCE assays except that the S-9 fractions were prepared in sucrose-phosphate buffer; cofactors, for 10 ml, were 24 mg of NADP and 45 mg of sodium isocitrate; and the remaining volumes consisted of culture medium--Fischer's medium with 5% heat-inactivated serum for the L5178Y assays, or McCoy's 5a medium containing 2.5% fetal calf serum, 2 mM L-glutamine, and 1% penicillin-streptomycin solution for the SCE assays.

Saccharomyces D3 Recombinogenic Assay

A stock culture of *S. cerevisiae* D3 is stored at 4°C.

For each experiment, broth containing 0.05% $MgSO_4$, 0.15% KH_2PO_4, 0.45% $(NH_4)_2SO_4$, 0.35% peptone, 0.5% yeast extract, and 2% dextrose was inoculated with a loopful of the stock culture and incubated overnight at 30°C with shaking.

The in vitro yeast mitotic recombination assay in suspension was conducted as follows. The overnight culture was centrifuged and the cells were resuspended at a concentration of 10^8 cells/ml in 67 mM phosphate buffer (pH 7.4). To a sterile test tube were added 1.00 ml of the resuspended culture, 0.50 ml of either the metabolic activation mixture or buffer, 0.20 ml of the test chemical, and 0.30 ml of buffer. The solvent used was DMSO. Several concentrations of the test emission samples (up to 5%, w/v or v/v) were tested in each experiment. For the positive control, 1,2,3-4-diepoxybutane was used.

The suspension mixture was incubated at 30°C for 4 hours on a roller drum. The sample was then diluted serially in sterile physiological saline, and 0.2 ml of the 10^{-5} and 10^{-3} dilutions was spread on plates containing the same ingredients as the broth plus 2.0% agar; five plates were spread with the 10^{-5} dilution and three plates were spread with the 10^{-5} dilution. The plates were incubated for 2 days at 30°C, followed by 2 days at 4°C to enhance the development of the red pigment indicative of adenine-deficient homozygosity. Plates containing the 10^{-3} dilution were scanned with a dissecting microscope at 10X magnification, and the number of mitotic recombinants (red colonies or red sectors) was recorded. The surviving fraction of organisms was determined from the total number of colonies appearing on the plates of the 10^{-5} dilution.

The number of mitotic recombinants was calculated per 10^5 survivors. A positive response in this assay is indicated by a dose-related increase of more than 3-fold in the absolute number of mitotic recombinants per milliliter and in the relative number of mitotic recombinants per 10^5 survivors.

Each extract of diesel or related environmental emission was tested at least twice on separate days. Prior to each assay, the samples were diluted in DMSO to form a series of concentrations that, when added to the cell/metabolic or buffer mixture, yielded the desired set of concentrations. The first experiment was a test over a wide range of concentrations to look for toxicity or recombinogenic activity. If no toxicity or recombinogenicity was observed, the second experiment was conducted at higher levels.

L5178Y Mouse Lymphoma Mutagenesis Assay

L5178Y mouse lymphoma cells, heterozygous for thymidine kinase, $TK^{+/-}$, were routinely grown as a suspension culture in Fischer's medium for leukemic cells of mice, supplemented with 31 μg/ml penicillin (1650 units/mg), 50 μg/ml streptomycin sulfate (F_o), 10% heat-inactivated horse serum, 0.1% Pluronic F68, and 0.22 mg/kg sodium pyruvate (F_{10p}). The cells were cleansed of homozygous ($TK^{-/-}$) cells with methotrexate. The cloning medium consisted of F_o with 20% heat-inactivated horse serum, 0.22 mg/kg sodium pyruvate, and 0.35% Noble agar. The selective medium contained 2.5 μg/ml TFT.

The positive controls were ethyl methanesulfonate (EMS), which induces mutagenesis without metabolic activation, and 3-methylcholanthrene (3-MCA), which induces mutagenesis only with metabolic activation. The negative (solvent) controls received 1% DMSO.

The results of dose-ranging assays were used to select 10 concentrations of each diesel emission sample for use in the mutagenesis assays that reflected cell survival in suspension culture ranging from 5 to 90% of the control level. In the mutagenesis assays, duplicate samples were used for each emission sample dilution and for the negative and positive controls. For each concentration of emission sample and for the controls, 6×10^6 freshly cleansed L5178Y $TK^{+/-}$ cells were exposed to 100 μl of a 100X concentration in 10 ml of medium in a sterile 50-ml centrifuge tube. The centrifuge tubes were rotated for 4 hours in a roller drum at 37°C. After the exposure, the cells were rinsed and then resuspended in 15 ml of F_{10p} in 50-ml centrifuge tubes for a final density of 4×10^5 cells/ml. The tubes were rotated in a roller drum for 2 days for expression of any mutations. Cell growth was monitored daily, and the cell concentrations were maintained at 3 to 15×10^5 cells/ml during the expression period.

The cells were then seeded in soft agar medium: 3×10^6 cells in cloning medium supplemented with 2.5 μg/ml TFT, and 600 cells in nonselective cloning medium to determine viability.

The mutation frequency was calculated by dividing the number of mutant cells per milliliter of original suspension culture by the number of viable cells per milliliter of original suspension culture. Therefore, the mutation frequency was the ratio of mutant cells to surviving cells at each test concentration.

An acceptable assay was one in which (1) the compound was tested to the level of approximately 10% total relative growth, (2) the relative plating efficiency of the solvent control was 60 to 110%, (3) the mutation frequency of the solvent control was no higher than 100 x 10^{-6}, and (4) the mutation frequency of the positive control was within acceptable limits based on historical data in the laboratory.

The test results were considered positive if the mutation frequency of at least one concentration was approximately twice that of the solvent control, or higher. A dose-response relationship was also a criterion; however, because sample availability was limited, this criterion was not always applied. The results were considered negative if the test was valid, but none of the treated samples showed a mutation frequency at least twice that of the solvent control.

Sister Chromatid Exchange Assay

Each SCE assay of the samples of diesel and related environmental emissions was conducted in duplicate, using Chinese hamster ovary (CHO) cells that were seeded at approximately 10^{6} cells per T-75 flask and grown for 1 to 2 days before exposure. The cells were grown in plastic T-75 flasks in an atmosphere of 5% CO_2 at 37°C in McCoy's 5a complete medium, which contains 15% fetal calf serum (FCS), 2 mM L-glutamine, and 1% penicillin-streptomycin solution. The emission samples were diluted serially in DMSO to form a series of concentrations that were further diluted 1:100 in culture medium. The final concentration of DMSO, therefore, was 1%, which is not cytotoxic.

The positive controls were 10^{-3} M EMS, a chemical mutagen that induces SCEs in the absence of a metabolic activation system, and 10^{-3} M dimethylnitrosamine (DMN), a compound that induces SCEs only with metabolic activation. The negative control was DMSO diluted in culture medium.

To test the coded samples, we modified the procedures developed by Perry and Evans (8) and Stetka and Wolff (13). In the assays performed without metabolic activation, the cultures were placed in McCoy's 5a complete medium containing 10^{-5} M BrdU, the coded sample at the desired final concentration was added, and the cells were grown in the dark at 37°C for 21.5 hours. Then the medium containing the sample dilution and BrdU was aspirated, the cells were washed two to three times with phosphate-buffered saline (PBS) at 37°C, and McCoy's 5a complete medium containing 0.4 μg/ml colchicine was added.

In the assays performed with metabolic activation, the cell cultures were exposed to the test sample in the metabolic activation mixture for 2 hours. (This shorter exposure time was used to prevent the occurrence of cytotoxic effects from the metabolic activation mixture.) After exposure, the medium containing the test sample and the metabolic activation mixture was aspirated, the cells were washed two or three times with PBS, and McCoy's 5a complete medium containing 10^{-5} M BrdU was added. The cultures were then incubated in the dark for 21.5 hours, after which colchicine was added to a final concentration of 0.4 μg/ml.

After 2.5 hours in colchicine, the mitotic cells were harvested. At harvest, medium (which might contain dividing cells) was removed and saved. PBS was added and the flasks were shaken approximately 20 times until almost all the mitotic cells were detached. The cell suspension in pooled medium and PBS was centrifuged, the supernatant was aspirated, and 4 ml of a hypotonic solution of 0.075 M KCl was added to the cells for 7 to 20 minutes at 37°C. The cells were resuspended in a fixative of methanol:acetic acid (3:1) three times, and air-dried slides were prepared. The cell density on a slide was monitored using a phase-contrast microscope, and at least two slides of appropriate cell density were made from each sample. The slides were stained by following a modification of the fluorescence-plus-Giemsa (FPG) technique (10), then examined to determine whether the metaphases had divided one (M_1), two (M_2), or three (M_3) times during exposure to BrdU. The concentrations of each test sample selected for cytogenetic evaluation were the five highest concentrations that permitted enough cells to divide twice so that SCEs could be enumerated. For each test, two cytogeneticists each analyzed 25 cells from coded duplicate samples for each of the five concentrations and from the positive and negative controls. Thus, 50 cells were evaluated per concentration.

The mean SCE frequency per cell and the mean SCE frequency per chromosome for each concentration of the test substance and for the controls was determined. Confidence limits were set by determining the standard error of the mean (assuming a Poisson distribution). These means were then evaluated by a one-way analysis of variance. The mean SCE frequencies per chromosome and per cell that each cytogeneticist observed for the same treatment group (variance within sample) were compared with the mean SCE frequencies observed for the other treatment groups and for the negative control (variance between samples). An F test was then performed to determine whether the between-sample variance (the effect of the chemical) was significantly greater than the within-sample variance (the variance between observers).

An acceptable assay was one in which (1) the maximum concentration used yielded sufficient M_2 metaphases for analysis, was the solubility limit, or, because of limited availability of sample, was 500 μg/ml; (2) the SCE frequencies in the positive controls were at least twice the negative-control SCE frequencies, and (3) SCE frequency analyses by at least two cytogeneticists were in general agreement.

A sample was considered to have elicited a positive response in the SCE test if the variance between treatment groups was significantly greater ($P < 0.05$; two-tailed test) than the variance within treatment groups for either SCEs per chromosome or SCEs per cell. A chemical was considered negative in the SCE test if the variance between treatment groups was not significantly different from the variance within treatment groups. When a weak SCE response to a sample was suspected but was not statistically significant, one or two additional cytogeneticists were asked to observe the same coded slides. The mean SCE frequencies determined by the three or four observers were then compared by a one-way analysis of variance, and the same criteria were applied to determine a positive or negative response.

RESULTS AND DISCUSSION

Saccharomyces D3 Recombinogenic Assay

The heavy-duty (Caterpillar) diesel emission was not tested in this system. For the other seven emission samples, in several experiments a slight elevation was observed in the number of recombinants at one or two concentrations. However these results were neither reproducible nor dose-related. We therefore conclude that none of the diesel and related environmental emission samples that were tested produced a recombinogenic effect in the yeast *S. cerevisiae* D3 assay. The concentration ranges used for the different samples are listed in Table 1.

TABLE 1. RESULTS OF *SACCHAROMYCES CEREVISIAE* D3 RECOMBINOGENIC ASSAY

Sample	Concentration (μg)	Toxicity
Coke oven emission	100 to 1,000	Toxic
Roofing tar emission	100 to 1,000	Toxic
Cigarette smoke condensate	10 to 5,000	Not toxic*
Gasoline Mustang emission	100 to 1,500	Not toxic*
Diesel Nissan emission	100 to 1,000	Toxic
VW Rabbit emission	100 to 1,000	Toxic
Diesel Oldsmobile emission	100 to 2,000	Toxic

*Because of the limited sample, a toxic dose could not be reached.

Simmon (17) reported that the *S. cerevisiae* D3 mitotic recombination assay is about 54% accurate in detecting chemical carcinogens as mutagens. All of 20 ultimate carcinogens tested in this study elicited a positive response, but only 18 of 49 procarcinogens increased the frequency of mitotic recombination. It is interesting that none of 7 polycyclic aromatic hydrocarbons (PAHs) requiring activation, including benzo(a)pyrene (BaP), was active in this assay (17). Failure to detect this class of mutagens could be due to inability of the compound to cross the yeast cell wall, or the recommended protocol used (1) may not be adequate because the organisms are exposed to the test chemical for a limited amount of time, during which no active cell growth occurs. We are currently investigating alternate protocols to increase the sensitivity of the assay.

L5178Y Mouse Lymphoma Mutagenesis Assay

Figures 1-8 present the results of testing the eight samples of diesel and related environmental emissions in the L5178Y $TK^{+/-} \rightarrow TK^{-/-}$ mutagenesis assay. For all the reported data, the mutation frequencies of the solvent and positive controls were within expected ranges, based on previous experiments conducted in this laboratory.

All of the emission samples gave positive mutagenic responses both in the presence and in the absence of metabolic activation, using the criteria of the L5178Y mouse lymphoma TK

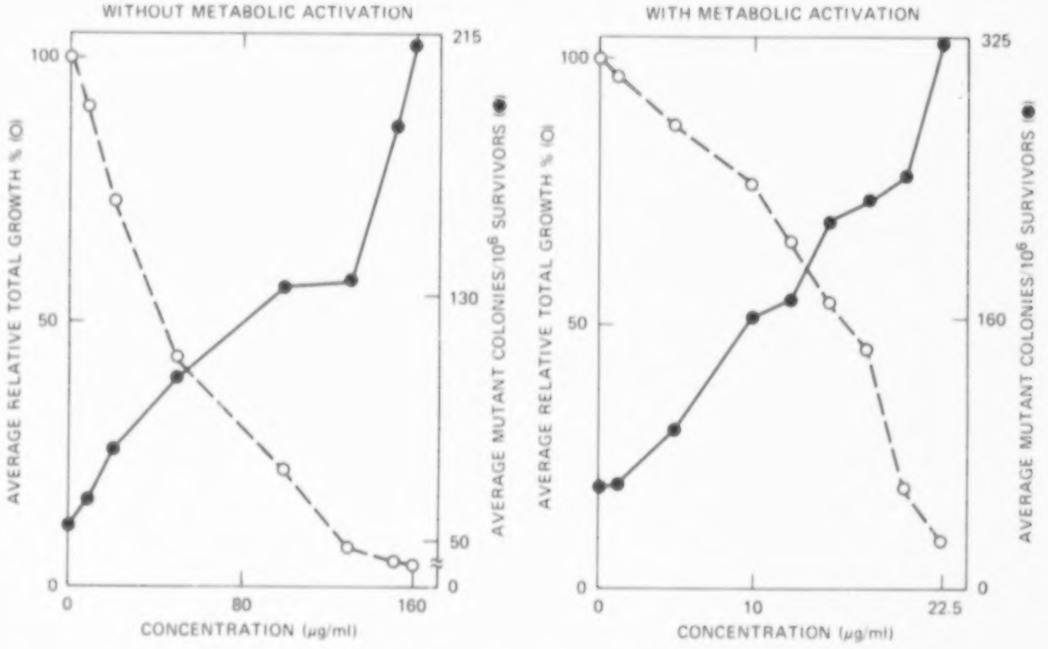


Figure 1. Results of the L5178Y Mutagenesis Assays of the Coke Oven Emission.

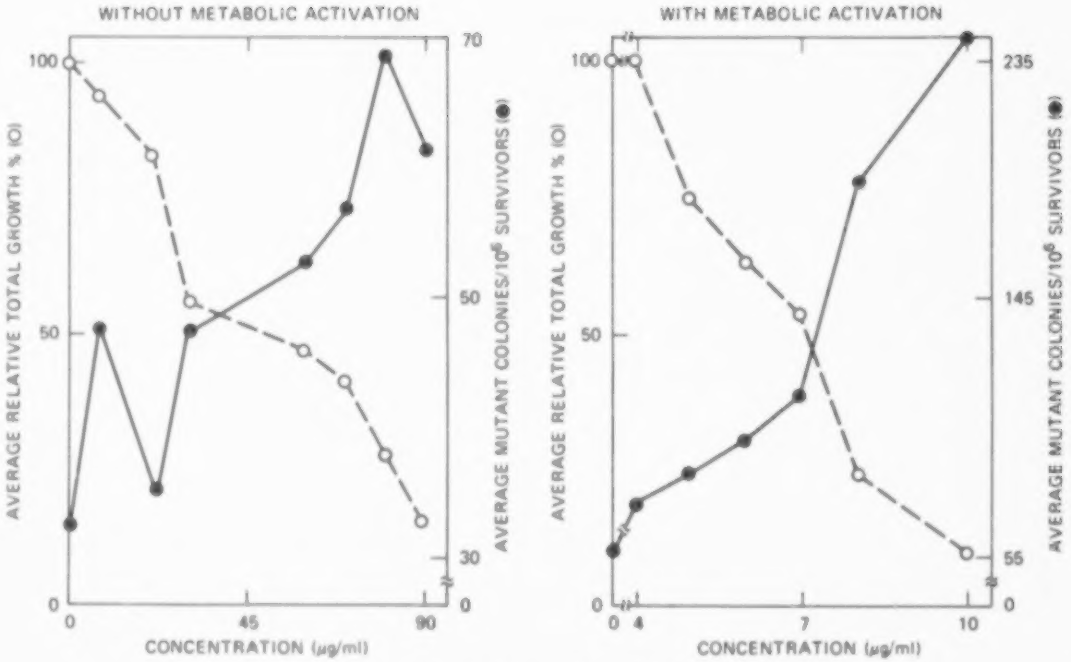


Figure 2. Results of the L5178Y Mutagenesis Assays of Roofing Tar Emission.

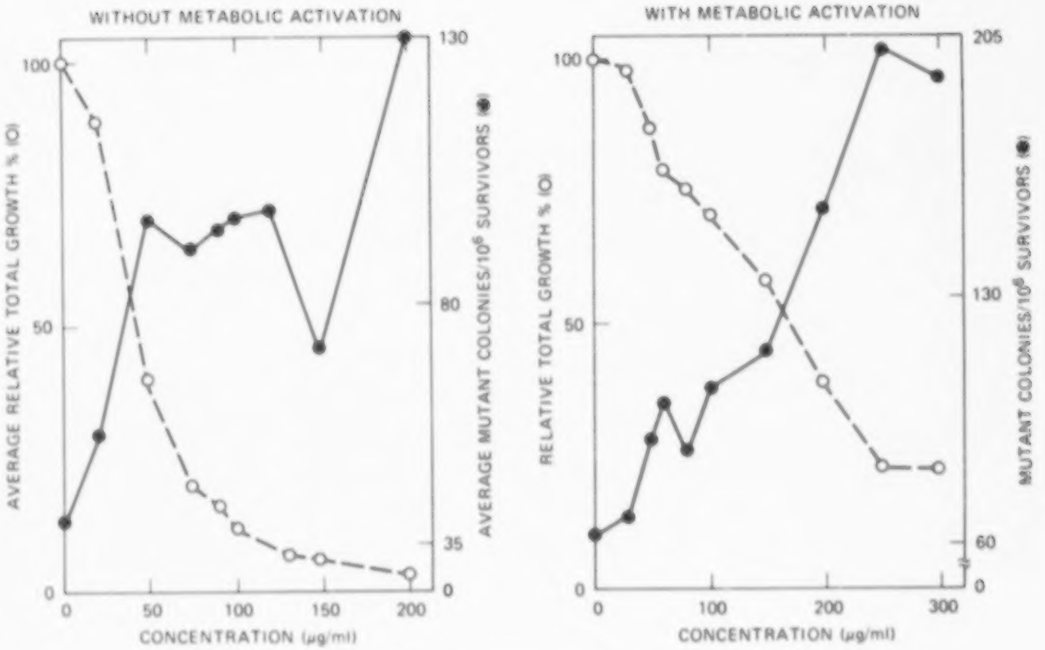


Figure 3. Results of the L5178Y Mutagenesis Assays of Cigarette Smoke Condensate.

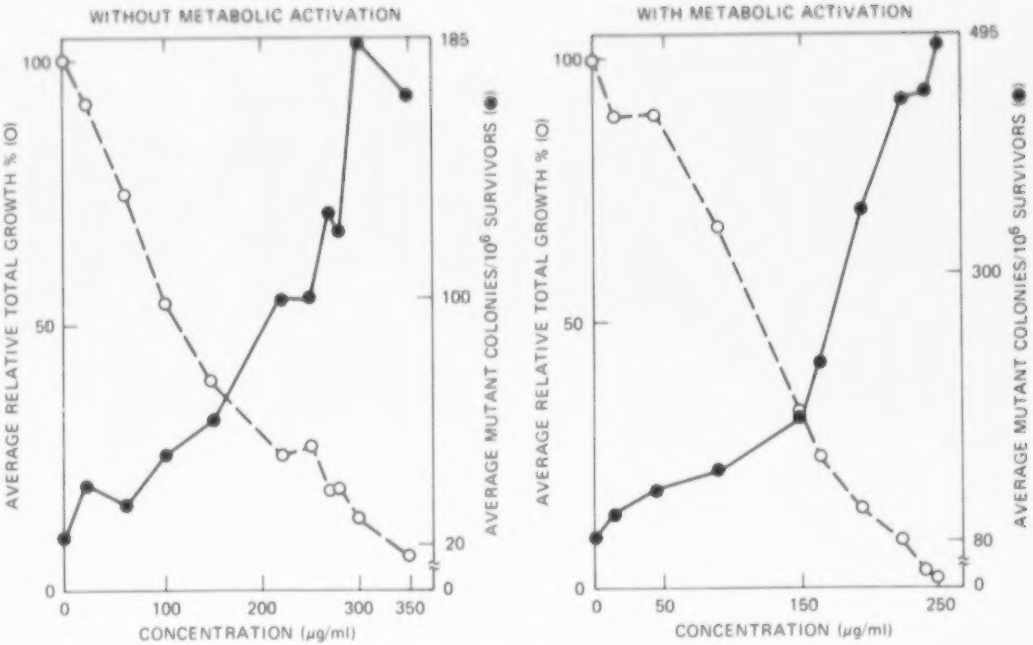


Figure 4. Results of the L5178Y Mutagenesis Assays of the Mustang Gasoline Engine Emission.

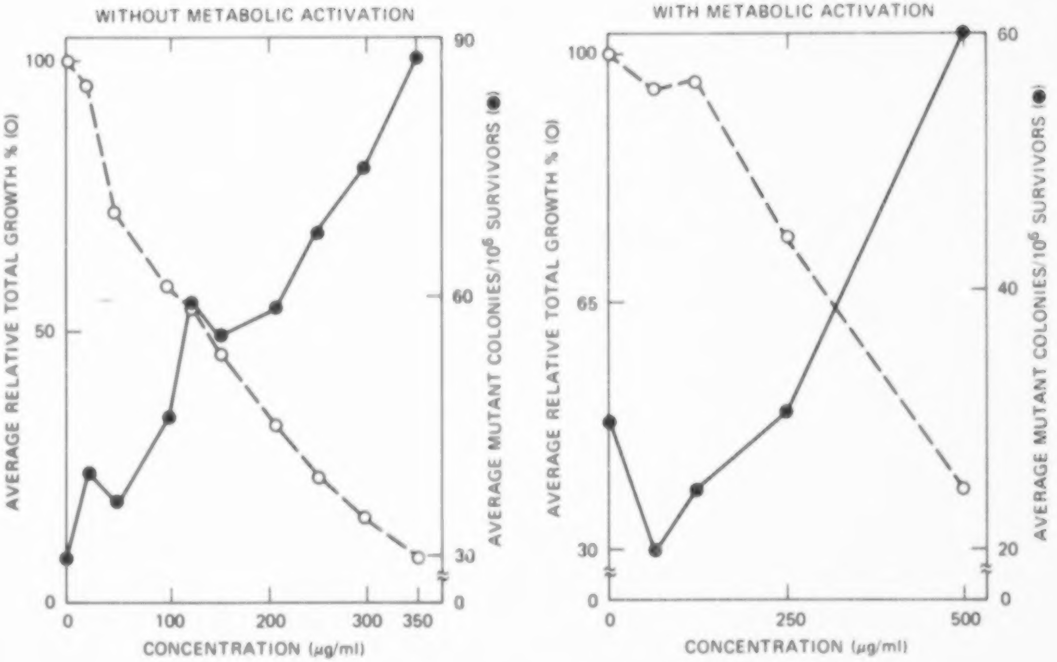


Figure 5. Results of the L5178Y Mutagenesis Assays of the Caterpillar Diesel Engine Emission.

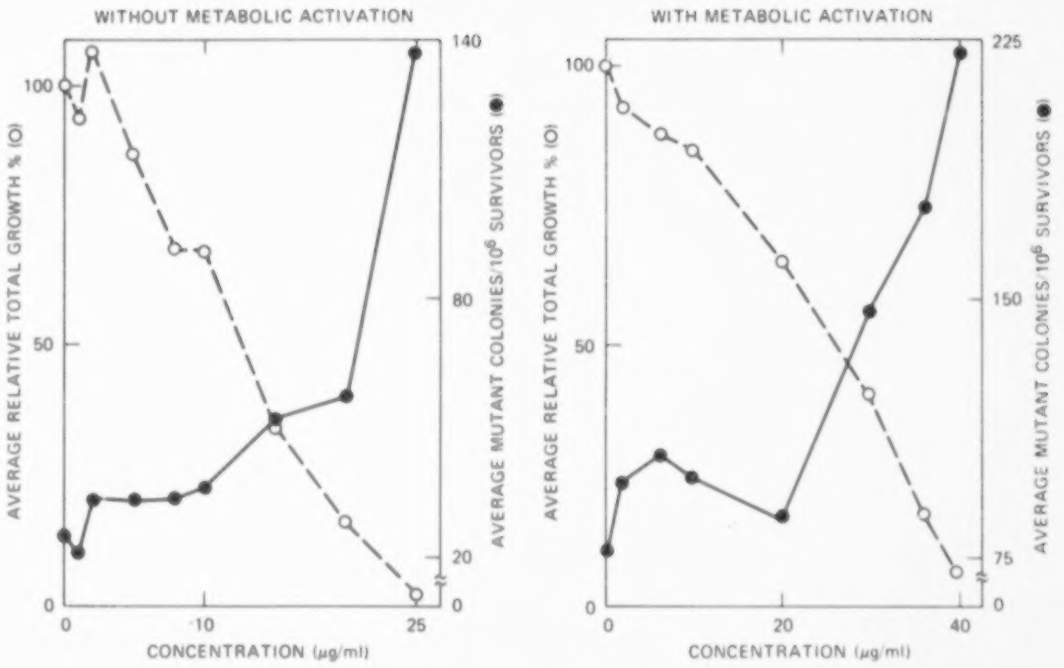


Figure 6. Results of the L5178Y Mutagenesis Assays of the Nissan Diesel Engine Emission.

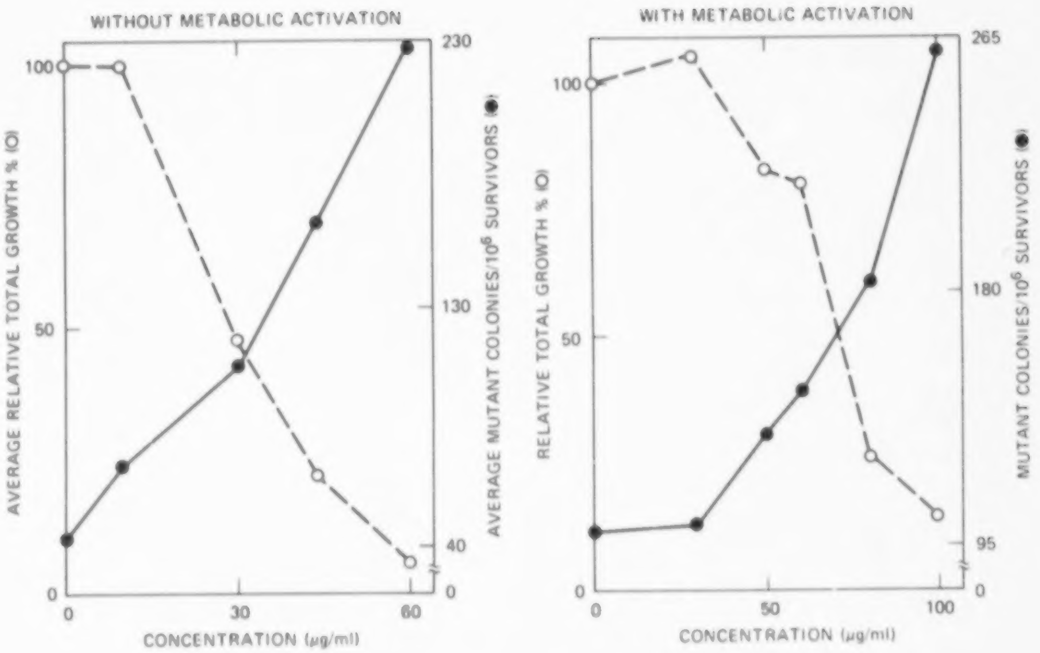


Figure 7. Results of the L5178Y Mutagenesis Assays of the VW Rabbit Diesel Engine Emission.

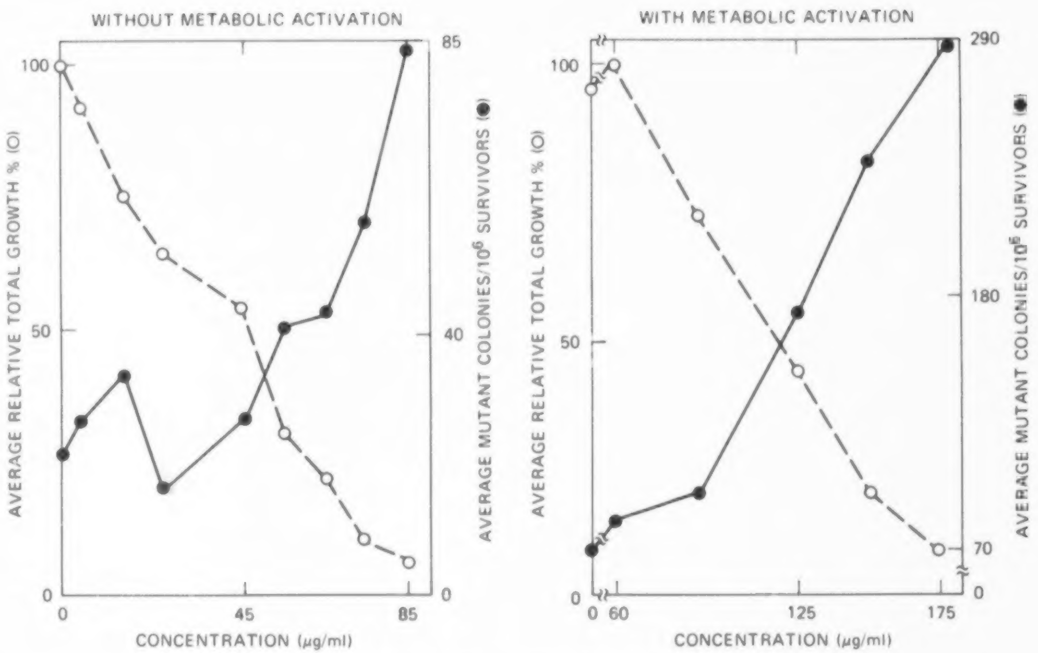


Figure 8. Results of the L5178Y Mutagenesis Assays of the Oldsmobile Diesel Engine Emission.

forward mutation assay. In comparison, mutagenicity in response to BaP has been detected in this assay only in the presence of activation. There were no unusual patterns in the cytotoxic or mutagenic responses. With cell survival levels of approximately 10%, the concentration producing the maximum mutagenic response was different for each sample. The four diesel engine emissions and the cigarette smoke condensate were more cytotoxic in the absence of metabolic activation than in the presence of activation. Examples of these results are illustrated by Figures 3 and 5-8. At cell survival levels of approximately 10% or greater, the maximum increases in mutation frequency occurred at concentrations ranging from 20 to 300 μg/ml. The mutation frequencies observed with these samples were 2 to 3.9 times the spontaneous frequencies.

Coke oven, roofing tar, and Mustang gasoline engine emissions were more cytotoxic in the presence of metabolic activation than in the absence of activation. Examples of these results are illustrated by Figures 1, 2, and 4. With cell survival levels of approximately 10% or greater, the maximum increases in mutation frequency occurred at concentrations ranging from 9 to 200 μg/ml. These samples yielded mutation frequencies 3.5 to 4.3 times the spontaneous frequency. Roofing tar and coke oven emissions were cytotoxic in the presence of metabolic activation at concentrations 7 to 10 times lower than those at which they were cytotoxic without activation.

Of the diesel engine emission samples tested, the Nissan diesel engine emission was the most cytotoxic. Of all the samples tested, the Mustang gasoline engine emission sample (both with and without metabolic activation) caused the highest increase in mutation frequency.

Sister Chromatid Exchange Assay

Figures 9-16 present the results of testing the eight samples of diesel and related environmental emissions in the SCE assay. Results of tests with metabolic activation on the sample from the emission of a Mustang gasoline engine are not presented because the CHO cultures were lost due to contamination in the first test with activation and there was not enough sample to repeat the test. All other samples were tested both with and without activation.

Figure 17 compares the responses of CHO cells exposed to diesel engine and related environmental emissions that resulted in significant increases in SCE frequencies without metabolic activation. Since these results are based on single experiments, minor differences in the responses to these samples at comparable concentrations should not be considered significant. With this caveat, we note that the coke oven emission sample caused the greatest increase in SCE

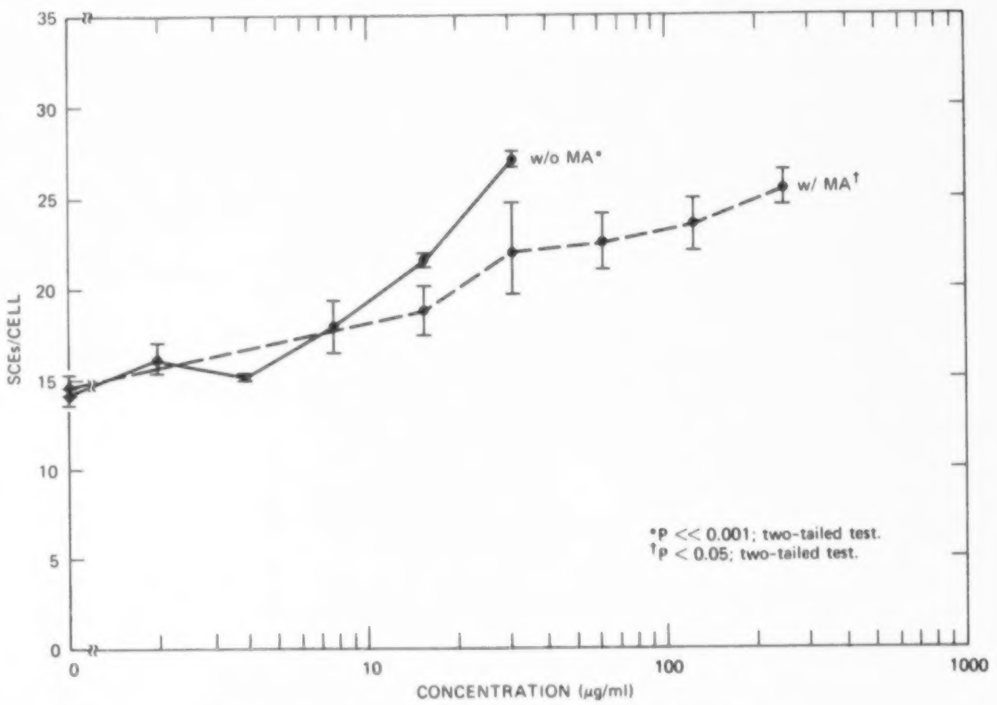


Figure 9. SCE frequencies in CHO cells exposed to Coke Oven Emission.

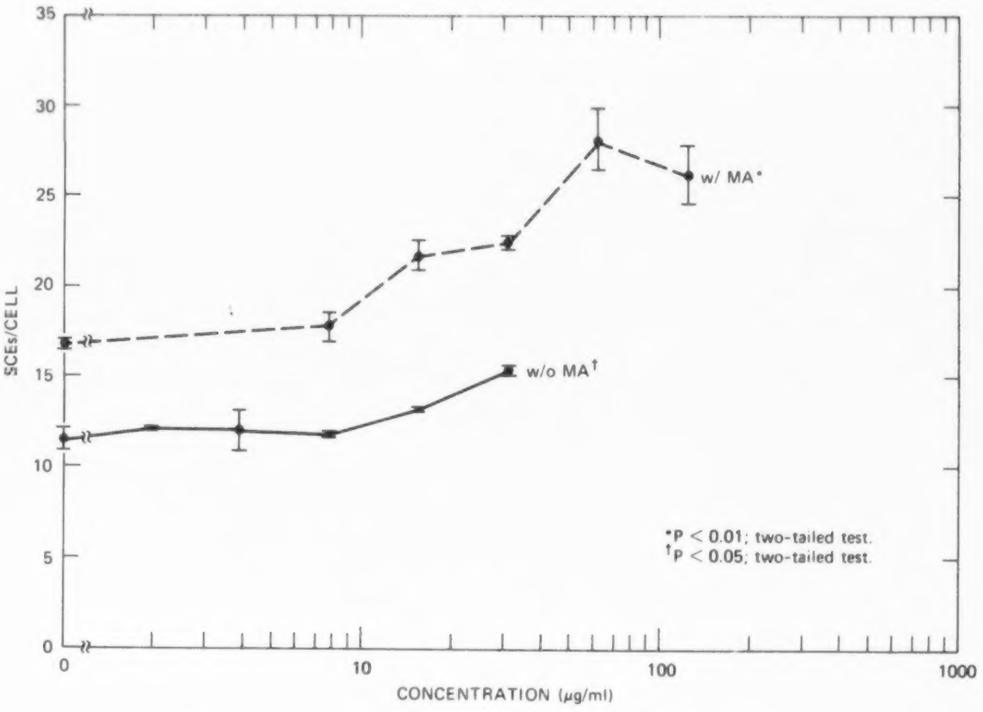
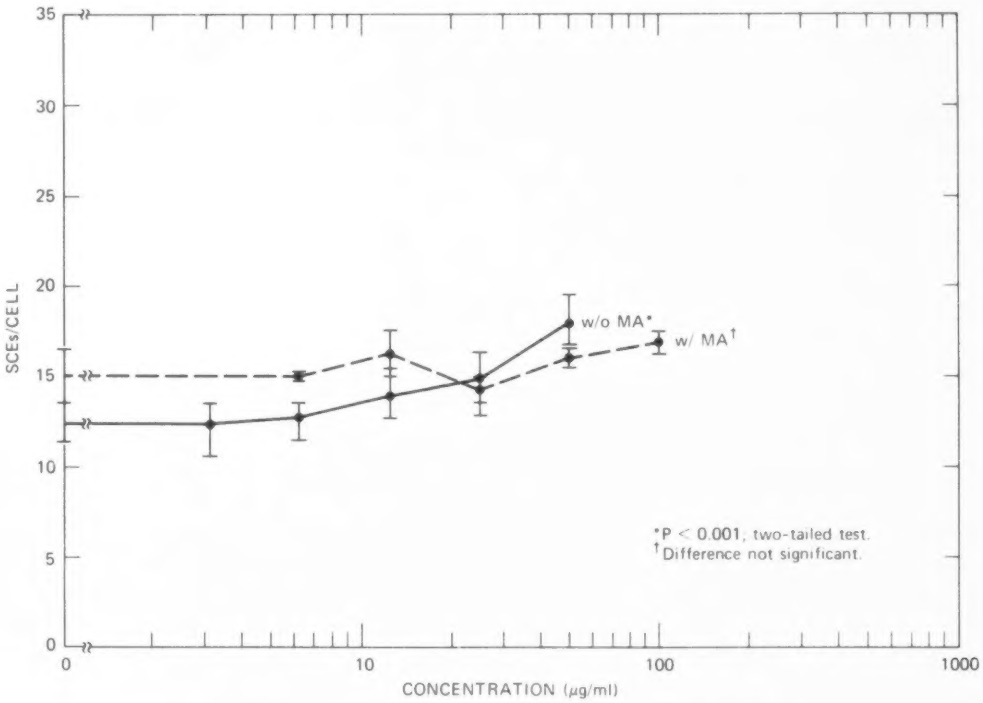


Figure 10. SCE frequencies in CHO cells exposed to Roofing Tar Emission.



*P < 0.001, two-tailed test.
†Difference not significant.

Figure 11. SCE frequencies in CHO cells exposed to Cigarette Smoke Condensate.

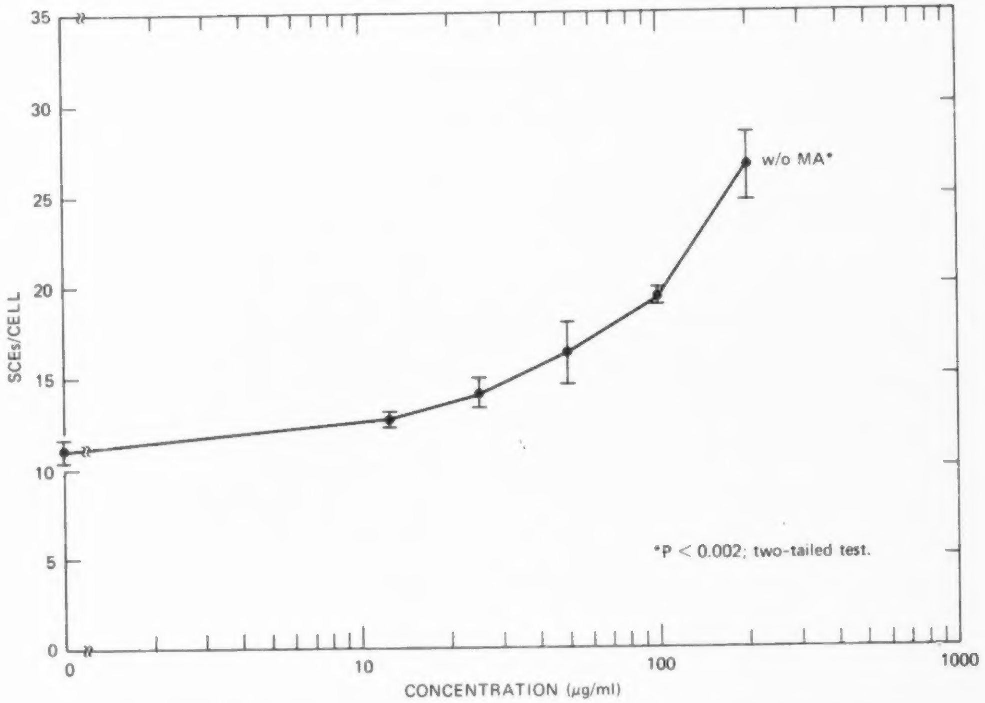
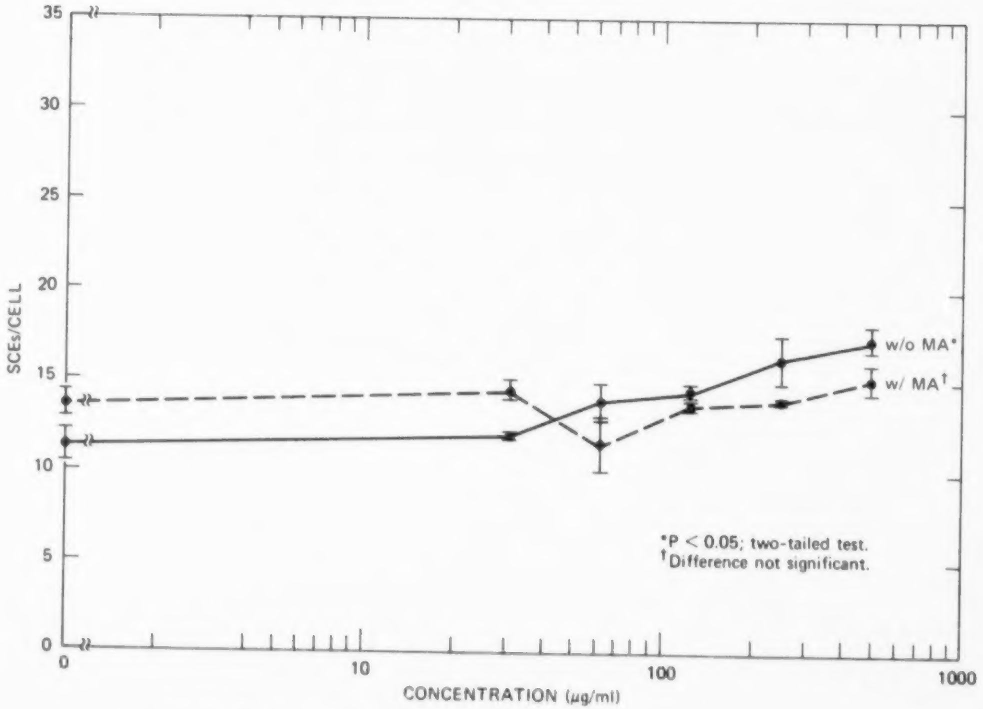


Figure 12. SCE frequencies in CHO cells exposed to Mustang Gasoline Engine Emission (this sample was tested only without metabolic activation).



*P < 0.05; two-tailed test.
†Difference not significant.

Figure 13. SCE frequencies in CHO cells exposed to Caterpillar Diesel Engine Emission.

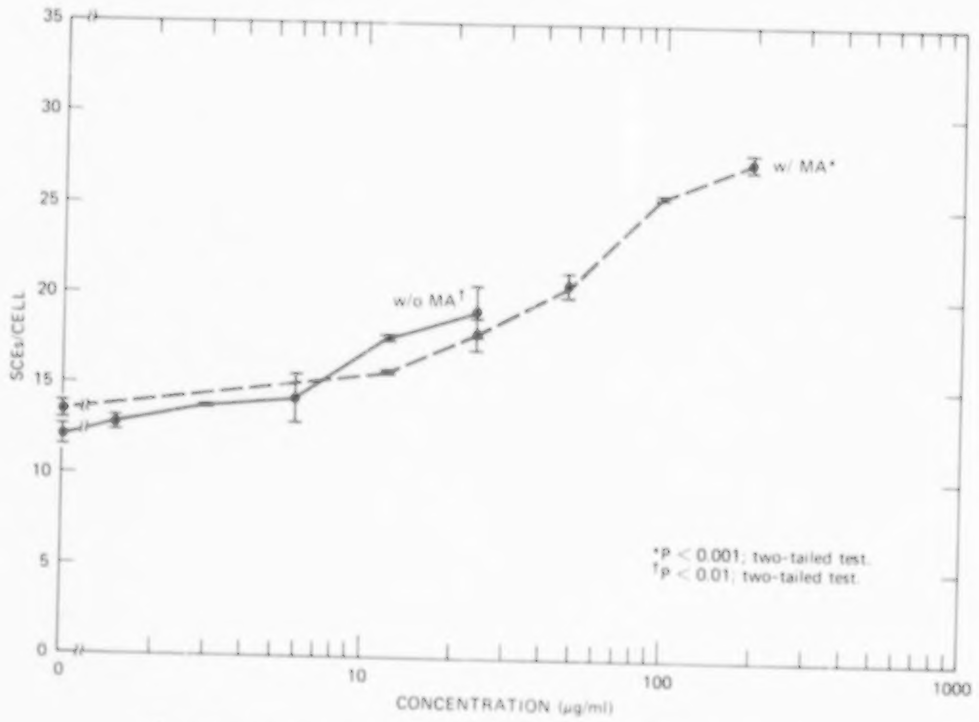


Figure 14. SCE frequencies in CHO cells exposed to Nissan Diesel Engine Emission.

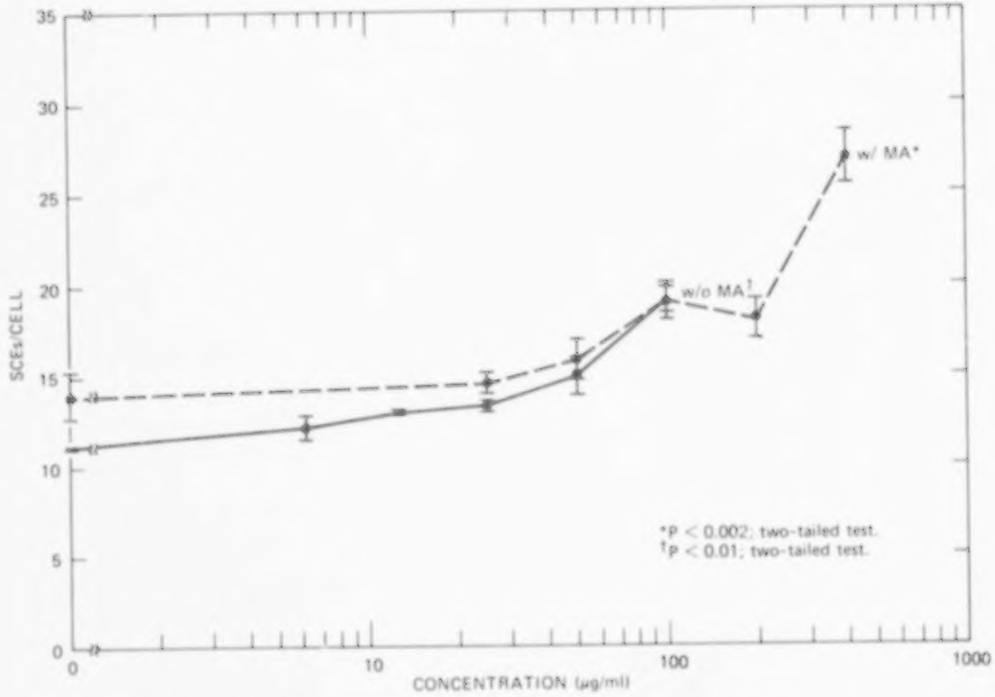
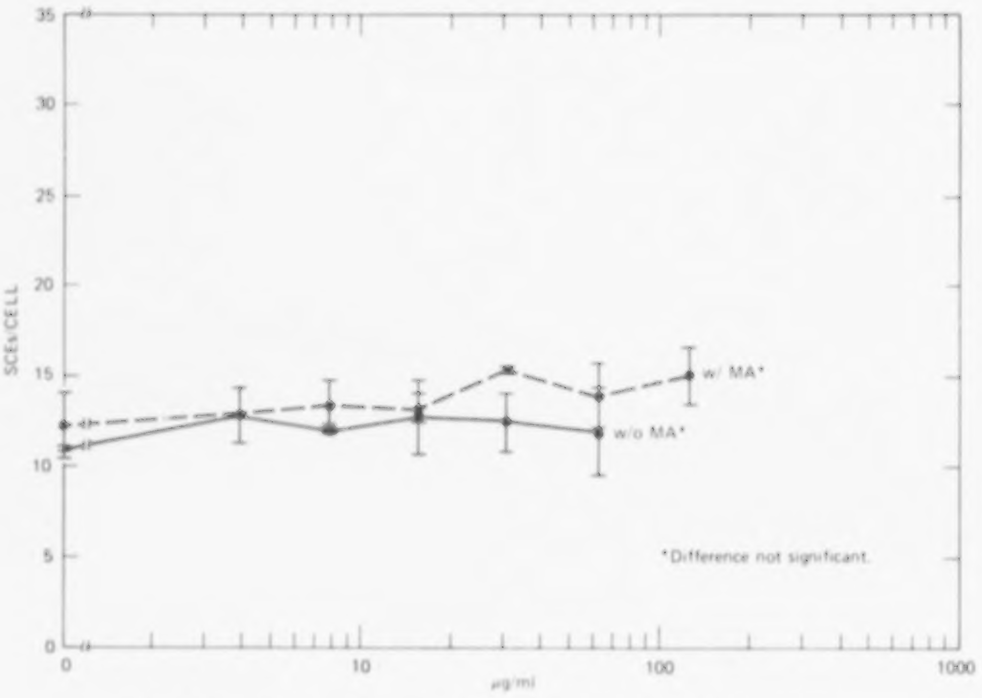


Figure 15. SCE frequencies in CHO cells exposed to VW Rabbit Diesel Engine Emission.



*Difference not significant.

Figure 16. SCE frequencies in CHO cells exposed to Oldsmobile Diesel Engine Emission.

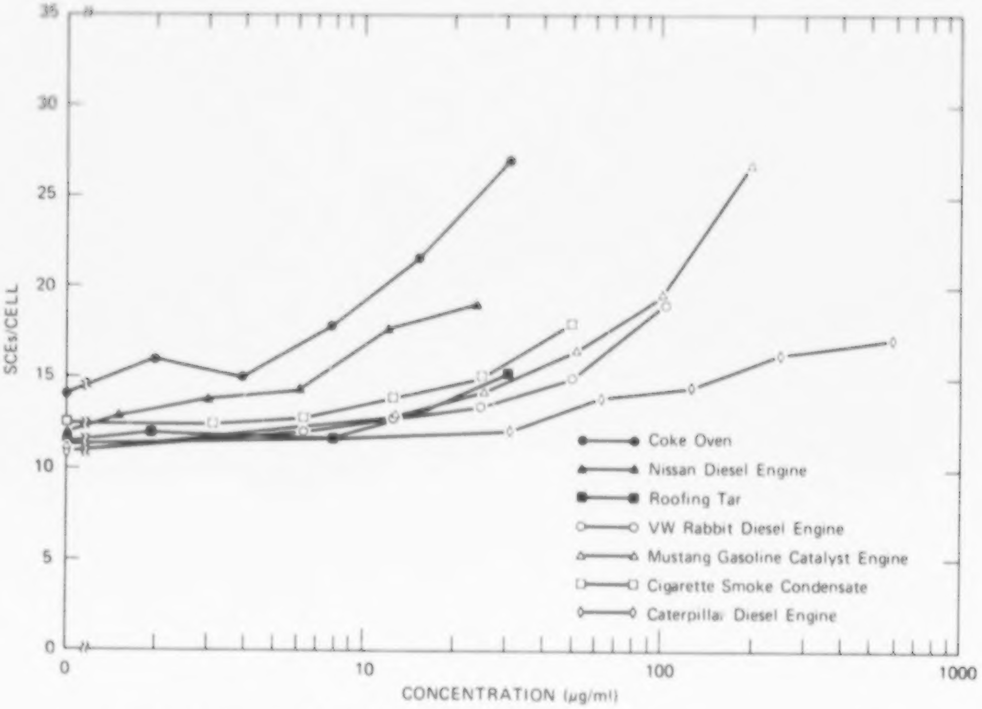


Figure 17. SCE frequencies in CHO cells exposed to Diesel and related environmental emissions without metabolic activation that yielded significant increases.

frequencies. The Nissan diesel emission sample seemed slightly more potent than samples of the cigarette smoke condensate, roofing tar emission, Mustang gasoline emission, and VW Rabbit diesel emission. The Caterpillar diesel sample was least effective in increasing SCE frequencies. Cytotoxicity also varied, the most toxic being the Diesel Nissan emission sample and the least toxic, the Caterpillar diesel emission sample.

Figure 18 presents the responses of CHO cells to diesel and other environmental emissions that resulted in significant increases in SCE frequencies with metabolic activation. For comparison, the results of testing BaP, a PAH that induces SCEs only with activation, are also included in this figure. These results are also based on single experiments, but BaP does appear to be more potent (and more cytotoxic) than the other samples. The VW Rabbit diesel emission sample was less active than the other samples at comparable concentrations. Because the SCE assays without metabolic activation involve a 21.5-hour exposure to the samples and those with metabolic activation involve only a 2-hour exposure, the relationship between cytotoxicity and chromosome damage as measured by SCE frequencies varies in the two assays. Direct comparisons of the results without attention to these relationships are, therefore, inappropriate. However, we did note that in contrast to BaP, which required metabolic activation, no emission sample was positive only with activation. This is consistent with the observations of Wang et al. (18), who found that the principal mutagens in city air (speculated to be derived from automobile exhaust), detected by the Ames Salmonella typhimurium assay, did not require activation by liver enzymes.

CONCLUSIONS

The absence of recombinogenic responses to the diesel and related environmental emission sample in the Saccharomyces D3 assay may be related to the short exposure time, during which no cell growth occurred, or it may be due to inability of the samples to cross the yeast cell wall.

Both the L5178Y mutagenesis and the SCE tests are in vitro mammalian assays in which the mutagenicity and/or DNA-damaging capabilities of PAHs such as BaP are detected only with metabolic activation. The failure of the SCE test to detect DNA-damaging effects of the cigarette smoke condensate and the emission from the heavy-duty (Caterpillar) engine with activation, whereas these samples induced mutagenesis in L5178Y cells with activation, may be related to the relatively short exposure time used for SCE testing with activation. It has been noted that all other tested samples induced SCEs

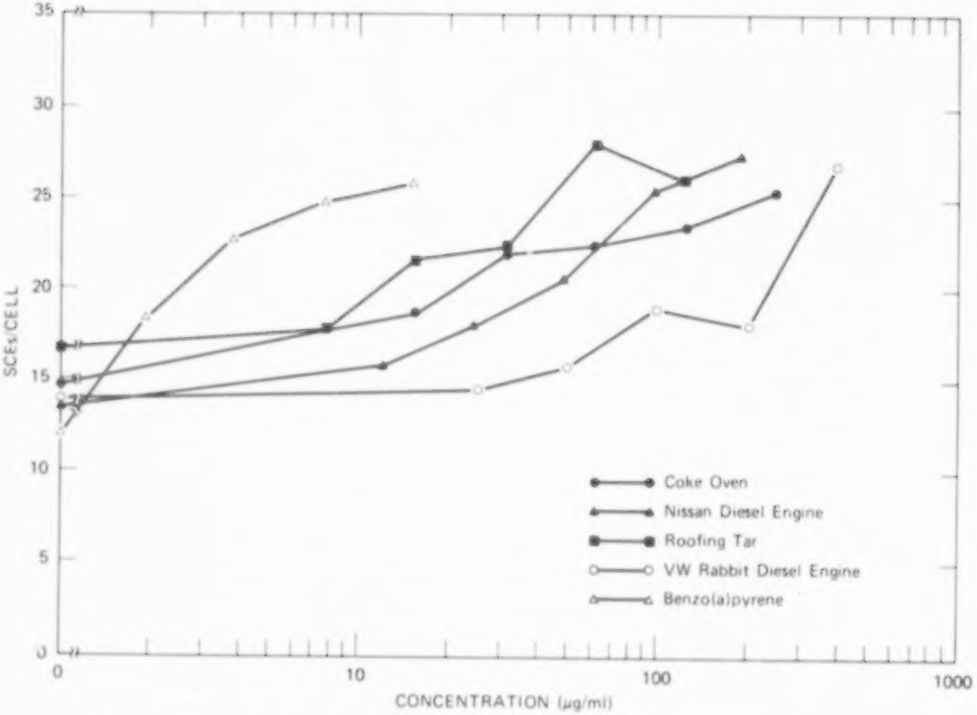


Figure 18. SCE frequencies in CHO cells exposed to Diesel and related environmental emissions with metabolic activation that yielded significant increases.

with activation, and all of the samples induced mutagenesis in L5178Y cells with activation.

The observations of increases in induced mutation frequencies in L5178Y cells following exposure to all of the emission samples in the absence of activation and of increases in SCE frequencies in CHO cells following exposure to all except the Oldsmobile diesel emission sample in the absence of activation indicate that, in contrast to BaP, the emission samples contain one or more substances that are direct-acting mutagens and chromosome-damaging agents. Further testing of various fractions from the emission samples using the L5178Y mutagenesis and SCE assays could assist in determining the identity of these direct-acting mutagenic and DNA-damaging agents.

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General Discussion

D. BRUSICK: That is very interesting data. Could you give a relative potency ranking to the mouse lymphoma the mouse data for various sample source?

A. MITCHELL: We feel very leery about coming up with any sort of calculations as to relative potency for any of these.

D. BRUSICK: Well in the mouse lymphoma it looked like they were very close together, in contrast to SEC's and certainly in contrast to the salmonella tests.

A. MITCHELL: Obviously the benzopyrene was the most positive of the mouse lymphoma. The diesel Olds was not very positive. We had to go pretty far out concentration-wise with the caterpillar fraction. I believe our greatest activities were in the coke oven and roofing tar complexes.

D. BRUSICK: It seemed that in the mammalian cells, in contrast to the salmonella data that the gasoline engine appeared to be more active than the diesel, which I find interesting, and maybe of importance in looking at this whole problem. If we have good solid positive data on mammalian cells, why are you trying to go back and regenerate more data from the yeast system which is another microbial assay if it isn't going to tell us anymore than we already know from salmonella and mammalian test data.

A. MITCHELL: You are an expert on yeast, but I think this is an EPA decision as to whether they want to keep going on with the matrix and look at the relative activities.

W. THILLY: I was very impressed with the completeness of the L5178 wide data. However, it seemed to me that, after looking at the data, the diesel sample was indeed very mutagenic to human cells in the presence of a metabolic activation system but in the absence of a metabolic activation system (up to 200 micrograms per ml) - no activity was seen. So as to your hypothesis, I would like to turn it over; and if there were differences at the cell level, it may be a matter of inactivation in possibly the L578 versus the human TK6.

M. WATERS: We have noted in tests with pesticide chemicals a number of cases where the pesticides are picked up in yeast systems, so I think from that point of view it is quite interesting to continue to look at the systems.

R. BILES: You may not be prepared to give these numbers, but I didn't get from your presentation the mouse lymphoma data for the diesel and the cigarette smoke condensate. Could you give the approximate concentration range with and without meatholic activation?

A. MITCHELL: Yes, I can, but it is in the notes.

P. SABHARWAL: I think he raised a very important question about the treatment of the S9 infraction. You can go up to only two hours before you have toxicity. We have resolved the toxicity problem by using a feeder layer so that we can now go up to 24 hours. In fact the freeder layer system is almost twice as active than that S9 infraction. Our recommendation is that those who are using S9 and are facing this problem of toxicity should consider using the feeder layers.

A. MITCHELL: This is an interesting approach. Using liver cells for cyto genetic assays has produced interesting data. A one-to-one correlation has not been found between the metabolic reactions in feeder layers or the hepatocyte and the S9 fractions. I think we are getting into another matrix of tests and suddenly this work becomes three dimensional instead of two, although I think it would be very interesting.

L. SCHECHTMAN: Actually one can circumvent problems with toxicity with an S9 preparation by prescreening the S9 as one prescreens serum lots and plastic dishes whatever you might use in a given bioassay. One can look for inherent toxicity of the S9 to the target cell and can screen the S9 for aryl hydrocarbon hydroxylase activity, epoxidase activity etc. This can a battery of tests useful in the selection of an S9.

A. MITCHELL: Correct, we do all of these things for the lymphoma assay. But quite frankly as to the chromatid exchange assay we do not do these things as routinely.

MUTAGENIC AND CARCINOGENIC POTENCY OF EXTRACTS OF DIESEL AND RELATED ENVIRONMENTAL EMISSIONS: IN VITRO MUTAGENESIS AND ONCOGENIC TRANSFORMATION

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ABSTRACT

Extracts from emissions of four diesel engines, a gasoline engine and three related environmental samples were tested in four *in vitro* assay systems designed to detect carcinogenic or mutagenic activity of chemicals. Samples from three of four diesel extracts, the gasoline engine, and all three related samples were positive in an enhancement of viral transformation assay. Two diesel samples, the gasoline engine extract and extract from coke oven emissions were positive for mutation induction in Chinese hamster ovary cells. Only the gasoline engine extract and the coke oven sample were positive in a DNA fragmentation assay using alkaline sucrose gradients. Experiments using chemical transformation of Syrian hamster embryo cells as an assay method have not been completed.

INTRODUCTION

Several *in vitro* tests for the identification of potential carcinogenic agents have been developed. Presently, the systems that correlate best with activity *in vivo* are mutagenesis assays in microbial (1) and mammalian (2) cells, mammalian cell transformation (3,4,5) analysis of DNA repair (3,6,7) or fragmentation (3,7,8), and enhancement of viral transformation (3,9,10,11). Four of the above test systems have been employed by us to evaluate the carcinogenic/mutagenic potential *in vitro* of extracts from diesel and related environmental emissions. These tests are: mutation assays in Chinese hamster ovary cells and the analysis of DNA fragmentation, chemical transformation, and enhancement of viral transformation in Syrian hamster embryo cells.

Mutagenesis assays in microbial systems have been reported to reliably detect 90% of known chemical carcinogens (1); a similar correlation has been established for the Chinese hamster ovary (CHO) mammalian cell mutation assay (2) used in the present study. Included in the latter study (2) were: direct acting alkylating agents, nitrosamines, heterocyclic nitrogen mustards, metals, polycyclic hydrocarbons, and ionizing and nonionizing radiation. In the group of 80 agents tested, the carcinogenic activity of 42 was known. The mutagenicity of this group showed a 95% correlation (40/42) with their carcinogenic activity *in vivo*.

The activity of chemical carcinogens in transformation assays *in vitro* with hamster embryo cells parallels their activity *in vivo*. Pienta et. al. (4) have tested various classes of chemicals including alkylating agents, polycyclic hydrocarbons, nitrosamines, aromatic amines and solvents and have found a 91% correlation with known carcinogenic activity (79/87); there were no false-positives. Casto et al. (3) have reported on the activity of 70 chemicals in Syrian hamster embryo cells using two assay systems. Combined data from focus and colony assays showed that 50/55 carcinogens were positive (91%), whereas none of the noncarcinogens or solvent controls induced transformants. Approximately 30 to 40 chemicals have been tested in common in two separate laboratories with good agreement between test results (12).

The close correlation between mutagenic and carcinogenic activity, for a large number of chemical compounds, has suggested that assays that directly or indirectly measure damage to cell DNA might be used to screen potential carcinogens or mutagens. These studies are based upon the methods used by McGrath and Williams (13) and Painter and Cleaver (14) for studying irradiation damage to the DNA of *E. coli* and Hela cells respectively. Many chemical carcinogens and mutagens induce DNA damage that is demonstrable following centrifugation alkaline sucrose gradients (10) or alkaline elution from filters (8). Casto et al. (3) have evaluated 77 compounds in Syrian hamster embryo cells for the capacity to induce DNA breakage. Of 55 carcinogens tested, 40 were positive (73%) following sedimentation in alkaline sucrose; there were no false positives among the remaining 22 chemicals. Using akaline elution techniques, 24 of 30 (80%) carcinogens caused breakage of DNA in V79 cells as reported by Swenberg et al. (8) and 63 of 70 (90%) carcinogens were positive in a more comprehensive study by Petzold et al. (15); again, there were no false-positives.

A different *in vitro* assay to detect potential carcinogens and mutagens has been described by Casto (11, 16) and Casto et al. (9,10,7) in Syrian hamster embryo cells. In this system, treatment of cells with carcinogenic or mutagenic chemicals results in an increased sensitivity to adenovirus transformation. Approximately 160 chemicals have been tested in this system with information available on 130 compounds regarding their current carcinogenic/mutagenic status. Of the known carcinogens or mutagens tested, 94% (83/88) were shown to significantly increase the transformation frequency of simian adenovirus, SA7, whereas only 1/42 noncarcinogens was positive (caffeine). Two of the false-negative compounds, when tested with an S-9 mix, converted to a positive response (urethane and dimethylnitrosamine).

BIOASSAY PROCEDURES

Mutagenesis Assays in CHO cells

Nearly confluent cultures of CHO cells (Clone K1-BHY Subclone D_1, obtained from A. W. Hsie, ORNL) were passaged to 100 mm dishes at 1 x 10^6 cells/plate. After 24 hr, 1.5 to 2 million cells per 100 mm dish were treated with increasing doses of test agents for 16 to 24 hours. Following chemical treatment, cells were collected by trypsinization and 10^5 cells were inoculated into 100 mm dishes. A minimum of ten dishes was used for each data point. Addition of medium to select for 6-thioguanine-resistant cells began 48 hours and 7 days after chemical treatment and continued for a period of 11 days. At this time, the cultures were fixed, stained with methylene blue, and the total number of mutant colonies enumerated.

For determination of cell survival, 100 to 200 treated or control cells were seeded into 60 mm dishes in F10 medium. After incubation for 9 days, the cultures were fixed, stained, and the total number of surviving colonies determined. The CHO cloning efficiency (CE) of treated cells was calculated as percent survivors relative to nontreated control cells. CE's were determined for cells replated at increasing post-treatment periods (i.e. 24-hr intervals).

Relative survival curves as a function of post-treatment times were calculated. Optimal chemical mutagen doses were determined from curves generated from mutagenesis experiments (*vide infra*).

If excess cell toxicity resulted from treatment with higher concentrations of compounds, the cultures were pooled and subcultured 3-to-5 days after treatment. To estimate survival and mutation frequency, treated or untreated cells were inoculated in 60 mm and 100 mm dishes as above and cultured for 9 or 11 days respectively. The cultures were fixed, stained and the total number of surviving and mutant colonies counted. A mutation frequency was calculated by determining the probability of the number of mutants among surviving cells.

Transformation Assays in Syrian Hamster Embryo Cell

Primary Syrian hamster embryo cell cultures (SHE) were prepared by trypsinization of eviscerated and decapitated fetuses after 13-14 days of gestation. Cells were resuspended in a Modified Dulbecco's medium (16) herein referred to as MDM, supplemented with 5% heat-inactivated fetal bovine serum (FBS), and 0.22g% $NaHCO_3$. Approximately 1.0×10^7 cells in 20 ml of medium were plated into 100 mm plastic Petri dishes and incubated in a 5% CO_2 atmosphere at 37°C. Total cell counts after 3 days usually ranged from 1.0-to-1.2×10^7 cells per plate. Secondary SHE were prepared by transferring 5×10^6 cells into 100 mm dishes in 20 ml of the above medium, but with 10% FBS.

For chemical transformation, stock solutions of extracts were prepared at concentrations of 1 or 10 mg/ml in acetone. Appropriate dilutions were made in complete medium to give the desired final concentrations. Evaluation of transformation was conducted by the focus assay method, adapted to SHE cells by Casto et al. (18). Secondary SHE were plated at 5×10^4 cells/60 mm dish in 4 ml of MDM containing 10% FBS and 0.11g% $NaHCO_3$. After 24 hr, 4 ml of chemical dilutions (as 2X concentrations) were added to each of 10-to-20 plates per dilution and incubated for 3 days at 37°C in 5% CO_2. The culture fluid was then replaced with fresh medium (0.22g% $NaHCO_3$) containing chemical dilutions at 1X concentrations and incubated for an additional 3 days. After a total of 6 days' exposure, the chemical-containing medium was removed and the cultures fed at 3-to-4 day intervals with 6 ml of MDM, 10% FBS, and 0.22% $NaHCO_3$. After 21-to-25 days from the time of chemical addition, the cells were formalin-fixed, Giemsa-stained and scored for transformed foci. Verification of the transformed morphology was made with a stereomicroscope at 10X to 30X magnification.

Cell lethality due to chemical was determined on plates seeded with 500 cells and treated as above. The surviving colonies were formalin-fixed and stained with 0.02% crystal violet approximately 8 days after seeding. The number of surviving colonies among cells treated with chemical was divided by the number of colonies in solvent-treated cells to give the surviving fraction of chemically treated cells.

Analysis of DNA Strand Breaks By Sedimentation in Alkaline Sucrose Gradients

Five to 35% sucrose gradients were prepared by 4 cycles of freeze-thawing (19) of tubes containing 18% sucrose in 0.05% EDTA which was adjusted to pH 12.6 with 10M NaOH. Primary hamster cell cultures, after 3 days' incubation, were passaged to 60 mm plastic dishes using 4 x 10^5 cells per dish. Immediately, or after 24 hr, the cells were pulsed with 0.5 μCi/ml of ^{3}H-labeled thymidine (^{3}H-TdR) for 24 hr. After ^{3}H-TdR labeling, all cultures were changed to MDM with 0.5% FBS and 0.22 g% $NaHCO_3$, and held for an additional 24 hr. At this time each dish contained approximately 10^6 cells. The test chemicals were prepared as 1-to-10 mg/ml stocks in acetone and added to prewarmed medium to give the desired final concentration. The SHE were treated for 18 hr, washed 1X, and removed from the dish with EDTA. Following centrifugation, the cells were resuspended in EDTA to give 10^5 cells per 0.1 ml. One-tenth ml of the cell suspension was then added to the top of a sucrose gradient tube layered with 0.3 ml of lysing solution (1% sarkosyl in 0.05% EDTA). The cells were then lysed at room temperature for 1 hr, placed in an SW-50 rotor, and centrifuged for 1 hr at 30,000 rpm in a Model L5-75B ultracentrifuge at 20°C. Three drop fractions were collected directly into scintillation vials following bottom puncture, neutralized with 1.0 ml of 0.2N HCl and prepared for counting by adding 5 ml of Bray's scintillation fluid to each vial. Counts were made in a Beckman LS-250 scintillation spectrometer, 10 minutes per sample. Data were plotted as percent of the highest count in each gradient. A shift in the peak of radioactivity greater than 3 fractions (more than 6 mm) from the control peak was considered positive evidence for chemical-induced DNA strand breaks (17).

Enhancement of Viral Transformation

Primary SHE were prepared by trypsinization of eviscerated and decapitated embryos as described previously. Total cell counts after incubation for 3 days usually ranged from 3.7-to-4.5 x 10^6 cells per plate. Stocks of simian adenovirus, SA7, were prepared in VERO cells by inoculation of cell cultures in 100 mm dishes at an input multiplicity of 2-to-3 plaque-forming units (PFU) per cell. Virus was adsorbed for 2 hr and 5 ml of medium (MEM, 5% FBS) then added to each plate. Cytopathic effects were complete by 72 hr, after which the infected cells were harvested, frozen-thawed 4X and virus separated from cell debris by low-speed centrifugation. Virus stocks for transformation assays were stored in 1 ml aliquots at -70°C.

Dilutions of test chemicals were prepared fresh each time by dissolving in acetone and appropriate dilutions then made in medium to give the desired final concentrations. In each experiment, 2 plates of SHE were treated with chemical for 18 hr prior to virus inoculation. Following incubation with the chemical, the SHE were rinsed with complete medium and inoculated with SA7. Transformation and clonal assays were performed with each treatment group as described below. Treatment of cells with chemical alone, using these protocols, did not result in detectable transformation.

A complete description of the methodology for adenovirus transformation has been presented elsewhere (20,21). Briefly, the procedure was as follows: SA7 was added to treated and control SHE (3-to-4 x 10^7 PFU/culture) and absorbed for 3 hr; the virus-inoculated cells were removed with trypsin (0.25% in MDM with 0.1 mM $CaCl_2$) then centrifuged and resuspended to 10^6 cells per ml in MDM, 10% FBS and 0.11 g% $NaHCO_3$. Cells were then mixed and plated into 60 mm dishes using 2 x 10^5 cells per dish; 3-to-4 ml of the above medium was then added to each plate. After incubation for 3 days, the medium was changed to MDM with 0.1 mM $CaCl_2$ (20,22), supplemented with 10% FBS and 0.22 g% $NaHCO_3$. At intervals of 4, 5 and 6 days, 3 ml of 0.5 g% Bacto-agar medium was added, and final focus counts made 25-30 days from the beginning of the experiment. All foci had the cellular and colony morphology representative for SA7 virus and have been shown previously to contain virus-specific SA7 "T" antigen. For survival assays, SHE were resuspended to 10^6 cells/ml and diluted in complete medium to give approximately 300 cells per 0.1 ml. Two-tenths ml (600 cells) was added to each of 5 plastic dishes, followed by 3 ml of MDM with 10% FBS and 0.11 g% $NaHCO_3$. Two to three days later, 3 ml of medium was added to each plate, and after 8-to-9 days' total incubation, the colonies were fixed in 10% buffered formalin and stained with 0.02% crystal violet. The cloning efficiency of virus-inoculated cells under these conditions was usually 8-to-15%. The number of colonies in five plates arising from chemically treated cells was divided by the number of colonies in five plates from control cells to give the surviving fraction of chemically-treated cells.

To determine the increase in transformation frequency (enhancement ratio) the number of SA7 foci counted from 10 virus-inoculated control plates, each receiving 200,00 cells, was used as the control transformation frequency and the total number of foci expected from 10^6 virus-inoculated cells was determined. On those plates receiving chemically treated cells, the frequency of SA7 foci per 10^6 cells was calculated by multiplying the actual number from five plates by the ratio: 1/surviving fraction of treated cells.

The enhancement ratio was determined by dividing the transformation frequency in treated cells by that obtained from control cells. For determination of statistical significance, a Table of Critical Ratios (16) was constructed from the Lorenz tables (23) which are based upon the Poisson distribution and which were originally designed to apply statistical tests of significance to virus plaque counts. The ratios used in the table are based upon the actual number of foci counted and not upon survival-adjusted values. An increased transformation frequency was considered statistically significant at the 5% or 1% confidence level if the enhancement ratio exceeded the appropriate value obtained from the Table of Critical Ratios.

RESULTS

All eight coded samples were tested in SHE cells in two to four separate experiments. When confluent cultures were exposed to the test samples, extracts from Caterpillar, Oldsmobile, Mustang and VW Rabbit were not overtly toxic at 500 μg/ml. Extracts from roofing tar were the most toxic (< 5% surviving cells at 100 μg/ml), followed by Datsun Nissan (25% survivors at 250 μg/ml), coke oven (50% survivors at 250 μg/ml), and cigarette smoke (40% survivors at 500 μg/ml).

When added to sparsely seeded cultures (1000 cells/dish), the dose sufficient to reduce cell survival by greater than 80% was significantly lower; approximately 100 μg/ml for Caterpillar, 50 μg/ml for VW Rabbit, 25 μg/ml for Oldsmobile, roofing tar, cigarette smoke, and Mustang, 6 μg/ml for Datsun Nissan, and 3 μg/ml for coke oven.

Data for enhancement of adenoviral transformation by the extracts of the various test samples are shown in Table 1 and Figure 1. With exposure to each sample, an absolute increase in the number of virus-transformed foci per plate

TABLE 1. ENHANCEMENT OF VIRAL TRANSFORMATION BY ORGANIC EXTRACTS FROM OLDSMOBILE AND DATSUN NISSAN DIESEL EMISSIONS

Sample[a]	µg/ml	Transformation[b] frequency	Enhancement[c] ratio
Oldsmobile	500	98	1.51*
	250	96	1.48*
	125	68	1.05
	62.5	78	1.20
	31.2	73	1.13
	0	65	1.00
Datsun Nissan	500	0	0
	250	236	4.07
	125	125	2.16
	62.5	111	1.91
	31.2	88	1.52*
	0	58	1.00

[a] Extracts were dissolved in acetone and added to cultures of SHE 18 hours prior to addition of Simina adenovirus SA7. Transformation and survival assays were conducted as described in the text.

[b] Number of SA7 transformed foci per 2 x 10^6 surviving cells.

[c] Enhancement was determined by dividing the transformation frequency in sample-treated cultures bu that obtained in solvent-treated cultures. Underlined values were significant at the 1% level; values with asterisk(*) were significant at the 5% level.

and a positive dose-response was induced at nonlethal concentrations. The Caterpillar extract, although giving a slight positive dose response (Figure 1) and producing a 1.35- and 1.40-fold increase in foci at 500 and 1000 µg/ml respectively, was the only sample that failed to induce a significant enhancement at either the 5% or 1% level.

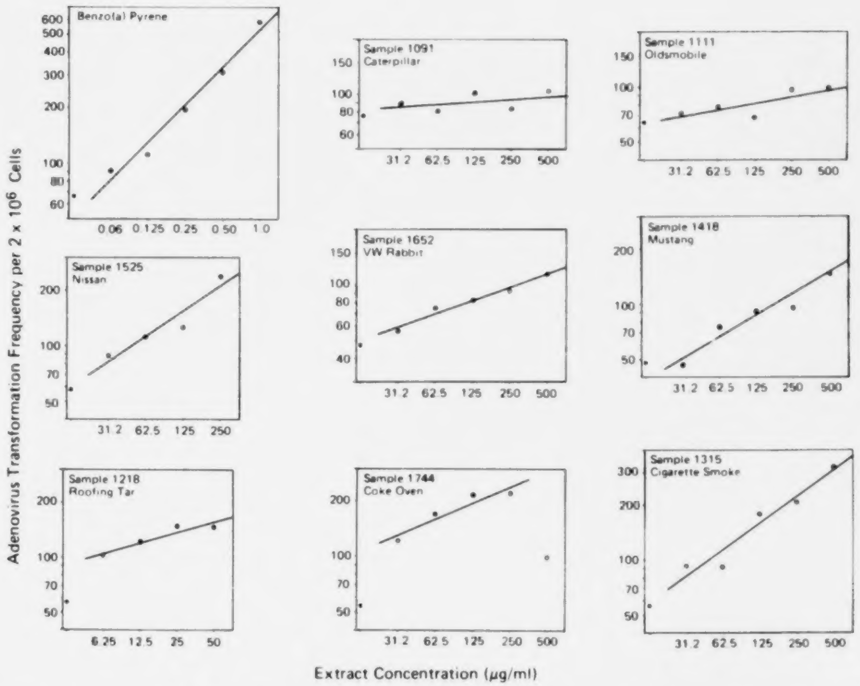

Figure 1. Dose response curves for extracts of diesel and related environmental emissions in the Syrian Hamster embryo-viral enhancement assay. Closed circles (●) represent the transformation frequency in cultures treated only with virus.

The Oldsmobile extract yielded a positive dose response (Figure 1) and a 1.48- and 1.51-fold increase in transformation frequency (Table 1); however, these increases at 250 and 500 μg/ml respectively were statistically significant at $p < 0.05$, but not at $p < 0.01$.

For comparison, the data for the Datsun Nissan extract are shown in Table 1. At 31 μg/ml, the increase in viral transformation is similar to that observed with 500 μg/ml of Oldsmobile extract. Dose response data for these and the remaining extracts are shown in the composite Figure 1.

If the various extracts are ranked according to the lowest effective concentration tested (LECT) that induces significant enhancement (Table 2), the roofing tar extract was found to be the most potent. Among the diesel samples tested, Datsun Nissan appeared to be the most potent followed by VW Rabbit, Oldsmobile and Caterpillar. The extract prepared from the gasoline-powered Mustang engine and the diesel powered VW Rabbit both induced enhancement at 125 μg/ml, but the Mustang extract was ranked higher since a greater increase in transformation frequency was obtained. None of the samples were as potent as B(a)P (positive at 0.125 μg/ml), but the dose-response curves generated by cigarette smoke and Datsun Nissan extracts were similar to that obtained for B(a)P (Figure 1).

Each of the organic extracts was tested in two to three separate experiments in CHO cells for the capacity to induce mutation as determined by resistance to 6-thioguanine. Cells were exposed for 24 hours and mutant selection was begun 48 hours or 7 days after treatment. The efficiency of mutant recovery was monitored with known 6-TG resistant cells and was determined to be 100% under the test conditions. Extracts showing a significant dose-mutation response relationship were from Datsun Nissan, VW Rabbit, Mustang, and coke oven; MMS was used as a positive control (Table 3). Graphic representations of the dose-response curves for extracts from the above emissions and the MMS control are shown in composite Figure 2.

Induction of cellular DNA breakage by the various extracts, proved to be the least sensitive of the three assays completed to date; only treatment with the Mustang and coke oven extracts caused breakage of SHE DNA in alkaline sucrose gradients. (Table 3 and Figure 3). The Mustang

TABLE 2. POTENCY OF ORGANIC EXTRACTS OF DIESEL AND RELATED ENVIRONMENTAL EMISSIONS FOR ENHANCEMENT OF VIRAL TRANSFORMATION IN HAMSTER EMBRYO CULTURES

		Enhancement[b]		
Sample	LECT [a]	LECT	HECT	Ranking[c]
Roofing Tar	3.1	1.63	2.58	1
Coke oven	7.8	1.69	4.04	2
Cigarette smoke	31.2	1.63	5.60	3
Datsun Nissan	62.5	1.91	4.07	4
Mustang	125	1.90	3.06	5
V.W. Rabbit	125	1.75	2.40	6
Oldsmobile	250	1.48*	1.51	7
Caterpillar	500	1.35	1.35	8
Controls:				
B(a)P	0.12	1.70	8.97	NA
MNNG	0.25	4.1	4.60	NA

[a] Lowest effective concentration tested that induced significant enhancement (P<0.01) of adenovirus transformation.

[b] Enhancement was determined by dividing the transformation frequency of extract-treated cultures by that obtained from solvent-treated cultures of SHE. HECT = maximal enhancement obtained at the highest effective concentration tested in these experiments. Asterisk (*) indicates values positive at the 5% level, but not at the 1% level of significance.

[c] Ranking was based upon the lowest concentration causing signigicant enhancement. Where two samples were positive at the same concentration, the one inducing more enhancement was ranked higher. NA= not included in ranking.

TABLE 3. INDUCTION OF MUTATIONS IN CHO CELLS AND DNA FRAGMENTATION IN SHE BY ORGANIC EXTRACTS OF DIESEL AND RELATED ENVIRONMENTAL EMISSIONS

Sample	Test system and response[a]	
	CHO Mutagenesis	SHE DNA fragmentation
Caterpillar	0(50-750)	0(30-250)
Oldsmobile	0(50-300)	0(30-250)
Datsun Nissan	+(25-100)	0(30-250)
V. W. Rabbit	+(100-400)	0(30-250)
Mustang	+(50-400)	+(250)
Roofing Tar	0(10-80)	0(30-250)
Coke oven	+(50-200)	+(125)
Cigarette smoke	0(50-200)	0(30-250)
Control:		
MMS	+(50-550)	+(12.5)

[a] A sample was considered positive in the CHO mutagenesis system if a significant dose response curve was generated over the dose range tested. In the DNA fragmentation system, a sample was positive if breakage occurred shifting the major peak of DNA greater than 6mm from the control DNA peak. Figures in parentheses show the dose range tested (nonlethal to ≈80% cell killing).

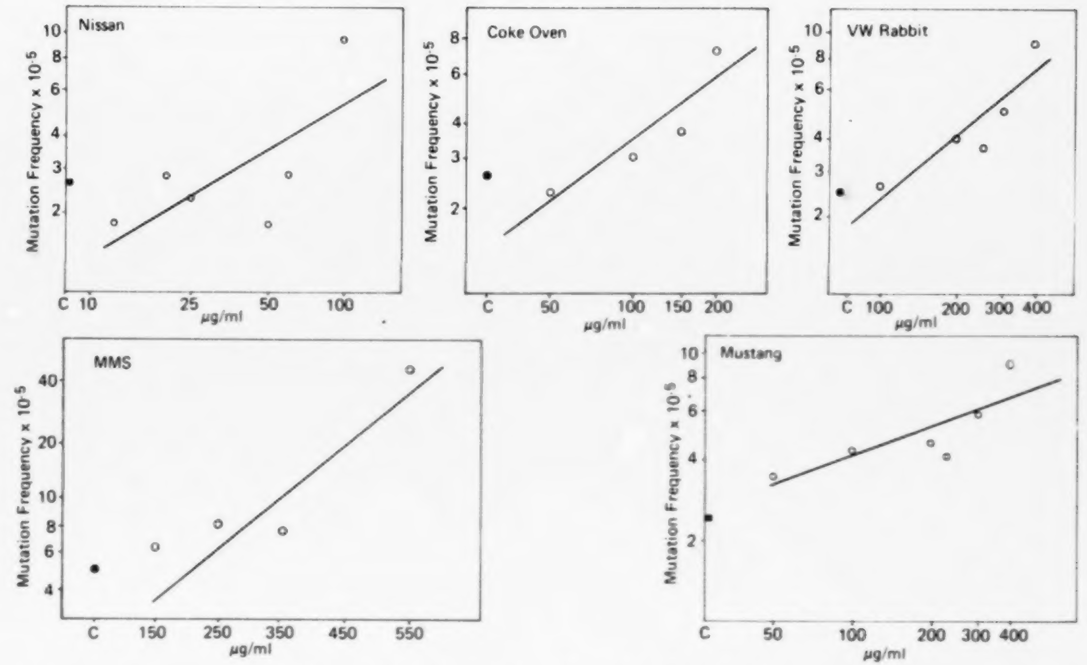

Figure 2. Dose response curve for extracts of diesel and related environmental emissions in the CHO mutagenesis assay. Closed circles (●) represent the mutation frequency in solvent-treated control cultures.

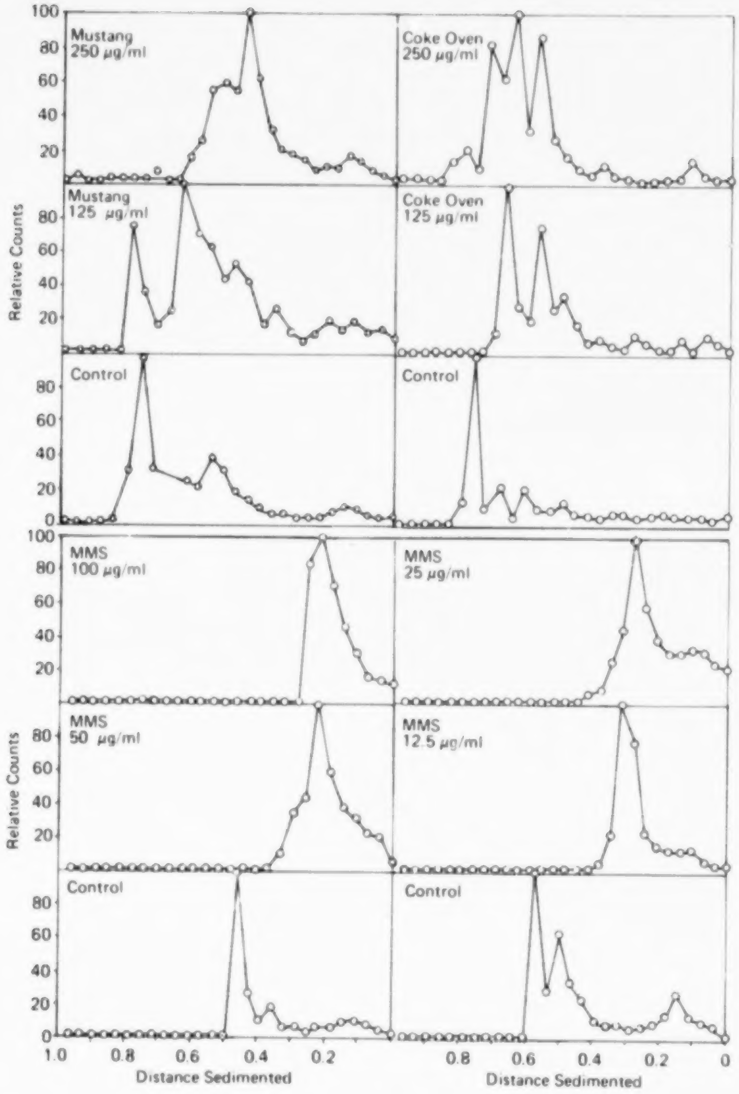

Figure 3. Alkaline sucrose gradients of DNA from Syrian Hamster embryo cells treated with MMS, coke oven or Mustang emission extracts. Sedimentation proceeded from right (top) to left (bottom).

extract was positive only at the highest dose tested (250 μg/ml), whereas the coke oven extract was positive at two dose levels (125 and 250 μg/ml); the positive control, MMS, caused breakage at 12.5 μg/ml and above (Figure 3).

At this time, the extracts from the different emission sources have been tested once for transformation of SHE cells by the focus assay method. B(a)P, the positive control, induced focus formation at concentrations between 0.06 μg/ml and 2.0 μg/ml (8 foci/60 dishes); the organic extracts were negative for transformation at doses from 1.5 to 100 μg/ml. Other experiments are in progress to verify these data and to attempt to increase the sensitivity of the system for these particular samples.

In summary, eight extracts from diesel and gasoline engine emissions were compared to extracts from related environmental emissions as coded samples in three *in vitro* assay systems. The viral enhancement assay produced a positive dose-response with all samples, however, the extracts prepared from Caterpillar emissions failed to induce a significant increase in the adenoviral transformation frequency. Only four of the eight samples caused a significant dose-response increase in 6-thioguanine resistant cells (Nissan, VW Rabbit, Mustang and coke oven); the remaining four (Caterpillar, Oldsmobile, roofing tar and cigarette smoke) caused an increase in mutation frequency only at the highest dose where approximately 70% or more of the cells failed to survive treatment.

Induction of cellular DNA breakage appeared to be the least sensitive of the three assays completed to date. Only the Mustang and coke oven emission extracts produced a significant change in the sedimentation profile of the DNA from treated SHE cells.

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General Discussion

B. CASTO: I would like to comment that the enhancement test actually measures the interaction of chemicals with cellular DNA. We can show, for example, that with chemicals there is an increased integration of viral DNA into cellular DNA after treatment, so it does depend in some way, on the interaction with cellular DNA. It doesn't necessarily have to be a direct interaction.

W. THILLY: That is really interesting. This assay has been of interest to many of us who think about the relationship between mutation and the induction of cancer. A parallel assay in prokaryotic systems is the induction of phage from the lysogenic to the lytic phase. Apparently some of these polycyclics or other mutagens somehow turn on the rec A protein. The rec A protein degradates the repressor on the lambd aphage. In other words, there is normally a repressor sitting on the OP binding site for the synthesis of the first protein necessary for lytic expressions in lamba.

B. CASTO: Yes, this is an extraneously added virus, not endogenous, but the general principle may be the same.

MUTAGENIC AND CARCINOGENIC POTENCY OF EXTRACTS FROM DIESEL RELATED ENVIRONMENTAL EMISSONS: SIMULTANEOUS MORPHOLOGICAL TRANSFORMATION AND MUTAGENESIS IN BALB/c 3T3 CELLS

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ABSTRACT

Particulate extracts from six different environmental emission sources were assayed for genotoxic activity in mouse BALB/c 3T3 Clone A31-1 cells. All compounds were tested simultaneously for both transforming and mutagenic potential with and without exogenous metabolic activation in the form of a 9000 x g post-mitochondrial hepatic supernatant fraction from Aroclor-1254 induced Fischer 344 rats. Dichloromethane particulate extracts from the exhaust of two light duty diesel engines (Oldsmobile and Nissan), one heavy duty diesel engine (Caterpillar) and one late model gasoline engine (Mustang II) were assayed in an identical manner to particulate extracts from the emissions of a roofing tar pot and a coke oven. No clear dose-dependent responses were observed, but several of the samples showed significant transforming and mutagenic activity. A qualitative ranking system showed the activity of these particulate extracts for either mutagenesis or transformation was: coke oven = Mustang II gasoline engine > Nissan diesel engine > roofing tar. Particulate extracts from the Oldsmobile diesel engine and the Caterpillar diesel engine showed essentially no activity.

INTRODUCTION

As various economic and environmental considerations force investigations of alternative fuels, assay systems must become available which are capable of detecting possible deleterious effects of exposure to the combustion products of various organic materials. The effluents produced during combustion of these fuels generally contain a myriad of chemicals, some harmless, some potentially genotoxic. A test system is therefore needed which is capable of detecting potentially genotoxic compounds in such mixtures. One assay system, which has shown its utility in detecting the genotoxic activity both of individual chemicals and complex mixtures (1,2,3, Schectman *et al.*, manuscript in preparation) has as an endpoint the morphological transformation of BALB/c 3T3 (3T3) mouse embryo cells (1,2). Mouse 3T3 cells are said to be "transformed" when they undergo certain morphological (1,3) and biological (1) changes that result in a phenotype similar to that expressed *in vitro* by tumor-derived cells. These *in vitro* transformed cells are generally assumed to be similar to malignant cells *in vivo* since transformed 3T3 cells cause tumors when injected into a syngeneic host (1). We have found that various drugs, pesticides, dyes, food additives, pre- and post-combustion products and industrial waste products can induce the transformation and/or mutation of 3T3 cells. Recently we have developed a mutagenesis assay for the BALB/c 3T3 cells in which we monitor the ouabain-resistance (OUA^r) phenotype (Schechtman *et al.*, manuscript in preparation). This assay has enabled us to simultaneously determine both the transforming and mutagenic activity of various compounds in one cell system. In this paper we show the results obtained when extracts of particulates collected from various emissions sources, including diesel engines, are tested for mutation and transformation of BALB/c 3T3 cells in the presence and absence of exogenous metabolic activation.

MATERIALS AND METHODS

CELL LINE

The BALB/c 3T3 (3T3) cells used in this study were from passages 12-18 of clone A31-1 established by Dr. Takeo Kakunaga (1).

CHEMICALS

Benzo(a)pyrene (BaP), NADH, NADPH, NADP, and ouabain were obtained from the Sigma Chemical Company St. Louis, MO. Acetone was obtained from the J. T. Baker Chemical Company, Phillipsburg, NJ, and dimethylsulfoxide (DMSO) was obtained from Fisher Scientific Company, Fair Lawn, NJ. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was purchased from the Aldrich Chemical Company, Milwaukee, WI. The various test articles were provided by the Environmental Protection Agency, Research Triangle Park, NC. Methods of collection and extraction of the test articles are presented elsewhere in this publication.

TRANSFORMATION AND MUTAGENESIS ASSAYS

Growth medium was Eagle's minimum essential medium (MA Bioproducts, Walkersville, MD) supplemented with L-glutamine (2.0 mM), non-essential amino acids (0.1 mM), penicillin (100 units/ml), streptomycin (100 ug/ml) and 10% heat-inactivated fetal bovine serum (Lot number 2910118, Flow Laboratories, McLean, VA). This lot of fetal bovine serum was previously checked for its ability to support growth of 3T3 cells in terms of cell generation time, plating efficiency, saturation density and maintenance of an even cell monolayer for at least five weeks.

In the mutagenesis and transformation assays with metabolic activation, 1-2 x 10^6 3T3 cells were suspended in a 4 ml reaction pool (pH 7.4) which consisted of phosphate buffered saline, 1.8 mM NADPH, 1.4 mM NADH, 7.4 mM glucose-6-phosphate and 1.9 mM NADP. To this was added 100 ul of a 9,000 x g supernatant (S-9) from the homogenized livers of Aroclor-1254 induced Fischer 344 rats (2), plus the appropriate amount of chemical to be tested. For assays performed without exogenous metabolic activation the reaction mixture consisted of 3T3 cells in 4 ml growth medium plus the chemical to be tested. The reaction mixture for either assay was incubated with continuous gentle agitation at 37° C for 2 hours in a humidified atmosphere of 5% CO_2 in air. The reaction was stopped by centrifugation (1000 x g, 4° C, 10 min). The cell pellet was washed once in Hank's balanced salt solution (MA Bioproducts, Walkersville, MD) and then suspended in growth medium. This final suspension was used as the source of cells for the cytotoxicity, transformation and mutagenesis assays.

For the transformation assays, cells were seeded at 1 x 10^4 cells/60mm dish and incubated for 4 weeks with medium changes twice a week. The cells were then fixed (methanol), stained (Giemsa), and examined for Type II and Type III morphologically transformed foci using the criteria established by Reznikoff et al., (3) for C3H 10T 1/2 cells. For the cytotoxicity assay, cells were seeded at a density of 250 cells per 60 mm dish. The cytotoxicity data was then used to calculate the number of cells at risk in the transformation assay.

For the mutagenesis assay, cells from the post-reaction suspension were seeded into 75 cm^2 culture flasks and grown for 48-96 hours to allow for expression of the mutagenic events. Cells were then collected by trypsinization and plated at a density of 2×10^5 cells/60 mm dish for selection of mutant colonies resistant to 1 mM ouabain (supplied fresh each week) according to the procedures of Baker et al., (4). Plating efficiency assays were run concomitantly with the mutagenesis assays so that mutation frequencies could be determined. Cells were fixed and stained after 7-9 days for determination of cloning efficiency and after 21 days for determination of the number of OUA^r cells.

RESULTS

In order to establish a baseline for the sensitivity of the test system in the absence or presence of exogenous metabolic activation, two model compounds were assayed. MNNG, a compound which is genotoxic for most micro-organisms and which does not require metabolic activation, was tested at seven different concentrations in the absence of metabolic activation (Table 1). MNNG induced Type II foci, Type III foci, and ouabain-resistant (Oua^r) mutants at several dose levels. Type III foci were observed at lower doses (0.03 ug/ml) than Oua^r mutants (0.3 ug/ml). A second chemical (BaP) was also assayed to determine the sensitivity of the system in detecting a compound which requires metabolic activation for its mutagenic/carcinogenic activity. Table 2 shows that both the transformation and mutation assays coupled with a source of exogenous metabolic activation could detect BaP at a level of 0.3 ug/ml. Data obtained with either chemical demonstrate a linear dose response. Linear correlation coefficients for MNNG were 0.92 and 0.97 for transformation and mutagenesis, respectively. Correlation coefficients were 0.95 for the transformation and 0.92 for the mutagenesis induced by doses of BaP between 0.01 and 3 ug/ml. The transformation and mutation frequencies induced by 10 ug BaP/ml were lower than expected and may reflect either the solubility of the compound or a differential response of the 3T3 cells with

TABLE 1

SIMULTANEOUS DETERMINATION OF THE CYTOTOXIC, MUTAGENIC, AND TRANSFORMING ACTIVITY OF MNNG TO BALB/c 3T3 Cl. A31-1 CELLS IN THE ABSENCE OF EXOGENOUSLY SUPPLIED METABOLIC ACTIVITY

			MORPHOLOGICAL FOCUS TRANSFORMATION				OUABAIN-RESISTANCE MUTAGENESIS		
TREATMENT[a]	DOSE (ug/ml)	CE[b]	#T-II[c]	#T-III[d]	TF[e] ($x10^{-4}$)	dwT-III/td[f]	#M[g]	MF[h] ($x10^{-6}$)	dwM/td[i]
MNNG	1.0	0.09	8	14	10.98	11/15	44	94.62	15/15
	0.5	0.10	4	2	1.40	2/15	17	29.51	10/15
	0.3	0.13	0	2	1.05	2/15	6	5.48	4/15
	0.1	0.15	1	0	<0.44	0/15	0	<1.16	0/15
	0.05	0.17	0	2	0.79	1/15	0	<1.04	0/14
	0.03	0.15	2	1	0.45	1/15	0	<0.85	0/15
	0.01	0.18	1	0	<0.39	0/14	1	0.70	1/15
DMSO	0.25%	0.22	1	0	<0.32	1/14	2	2.57	2/15

[a] Cells were treated ± test chemical in suspension for 2 hr at 37°C (see Materials and Methods)

[b] CE = Cloning efficiency, i.e., number of colonies per number of cells seeded in accompanying cytotoxicity assay

[c] T-II = Number of morphologically transformed Type II foci per condition

[d] T-III = Number of morphologically transformed Type III foci per condition

[e] TF = Transformation frequency ; number of Type III foci per viable cell seeded

[f] dwT-III/td = Number of dishes with Type III foci per total dishes

[g] M = Number of Oua^r mutant colonies per condition

[h] MF = Mutation frequency ; Number of OUA^r colonies per viable cell exposed to ouabain

[i] dwM/td = Number of dishes with OUA^r colonies per total dishes

TABLE 2

SIMULTANEOUS DETERMINATION OF THE CYTOTOXIC, MUTAGENIC, AND TRANSFORMING ACTIVITY OF BaP TO BALB/c 3T3 Cl. A31-1 CELLS IN THE PRESENCE OF EXOGENOUSLY SUPPLIED METABOLIC ACTIVITY

TREATMENT[a]	DOSE (ug/ml)	CE[b]	#T-II[c]	MORPHOLOGICAL FOCUS TRANSFORMATION			OUABAIN-RESISTANCE MUTAGENESIS		
				#T-III[d]	TF[e] $(\times10^{-4})$	dwT-III/td[f]	#M[g]	MF[h] $(\times10^{-6})$	dwM/td[i]
BaP + S-9	10	0.22	3	2	0.65	2/14	11	26.6	9/12
	3	0.23	5	4	1.23	4/14	50	83.4	14/15
	1	0.29	2	2	0.46	1/15	17	22.6	9/15
	0.5	0.27	0	1	0.24	1/15	6	7.3	6/15
	0.3	0.28	1	2	0.48	2/15	5	5.4	5/15
	0.1	0.28	1	0	<0.24	0/15	0	1.0	1/15
	0.03	0.31	1	0	<0.28	0/15	0	<1.4	0/15
ΔS-9 + BaP	10	0.22	7	2	0.65	2/14	6	18.7	6/12
S-9 + Acetone	0.25%	0.30	2	1	0.22	1/15	0	<1.0	1/15
Acetone	0.25%	0.22	2	0	<0.30	0/15	0	<0.9	0/15

[a] Cells were treated ± test chemical in suspension for 2 hr at 37°C in presence of an Aroclor 1254-induced rat (Fischer 344, male) hepatic 9000 x g supernatant (S-9) and an NADPH-generating system (see Materials and Methods)

[b] CE = Cloning efficiency, i.e., number of colonies per number of cells seeded in accompanying cytotoxicity assay

[c] T-II = Number of morphologically transformed Type II foci per condition

[d] T-III = Number of morphologically transformed Type III foci per condition

[e] TF = Transformation frequency; number of Type III foci per viable cell seeded

[f] dwT-III/td = Number of dishes with Type III foci per total dishes

[g] M = Number of ouabain-resistant mutant colonies per condition

[h] MF = Mutation frequency; number of OUA^r colonies per viable cell exposed to ouabain

[i] dwM/td = Number of dishes with OUA^r colonies per total dishes

TABLE 3

THE TOXICITY OF EXTRACTS FROM VARIOUS ENVIRONMENTAL EMISSION SOURCES IN THE ABSENCE OR PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

SOURCE OF EMISSION EXTRACT	ABSENCE		PRESENCE	
	DOSE RANGE TESTED[a] (μg/ml)	LOWEST TOXIC DOSE[b] (μg/ml)	DOSE RANGE TESTED[a] (μg/ml)	LOWEST TOXIC DOSE[b] (μg/ml)
Caterpillar Diesel Engine	10-300	200	10-300	200
Oldsmobile Diesel Engine	10-280	40	10-280	180
Roofing Tar	10-300	None	10-300	None
Nissan Diesel Engine	2.5-100	2.5	5-150	10
Mustang II Gasoline Engine	2.5-500	None	2.5-500	None
Coke Oven	10-1000	None	10-1000	None

[a] Seven doses were tested within the stated range.

[b] Less than 80% survival relative to the solvent control.

increasing BaP concentration. Such a decrease in the transformation frequency induced by high doses of polycyclic aromatic hydrocarbons has been observed by others (1).

Dichloromethane (DCM) extracts of particulates from diesel and related environmental emissions were tested at seven doses with and without a source of exogenous metabolic activation. Dose selection was determined by preliminary cytotoxicity assays. If the test article was toxic, a dose range was selected which included at least one toxic dose. If the compound was not toxic in the preliminary assay, it was tested through a range of 7 doses with an upper limit of 1 mg/ml. A further consideration used for dose selection was to have as many common doses as possible among the test chemicals. Table 3 lists the doses tested for each sample as well as the relative toxicity of the sample to 3T3 cells either in the presence or absence of exogenous metabolic activation. The three diesel DCM particulate extracts were all toxic at a minimum of one dose, while the three comparative samples showed no toxicity during the treatment period for the simultaneous mutagenesis/transformation assay. The addition of exogenous metabolic activation had no effect on the toxicity of the Caterpillar sample but reduced the toxicity of the Oldsmobile and Nissan diesel samples.

The results from the transformation and mutation assays with the six particulate extracts were less easy to interpret due to the lack of a clear dose response. In general, even though several individual doses of a particular DCM extract did induce a significant increase in either the frequency of transformed foci or OAU^r colonies, a dose-dependent increase in either transformation or mutation frequency was not found (see Discussion for further comments).

The majority of the DCM extracts were above their limit of solubility in the aqueous growth medium at all seven test doses. Therefore it may be reasonable to assume that all seven dose levels were, in fact, quite similar. With this assumption the data for all doses of a given DCM extract were combined, e.g., the total number of Type III foci observed for an individual extract were divided by the total number of surviving cells. This frequency was then considered as the resultant of the dose range as specified in Table 3. At the same time the data from the appropriate negative (solvent) control (one negative control as assayed concomitantly with each extract) were combined to give the historic background transformation and mutation frequencies for this assay series. A positive control of either 1.0 ug MNNG/ml for assays in the absence of exogenous metabolic activation or 12.5 ug BaP/ml for assays in the presence of exogenous metabolic activation was tested simultaneously

TABLE 4

TOTAL GENOTOXIC EVENTS OCCURING AFTER EXPOSURE TO VARIOUS DOSES OF EMISSION EXTRACTS IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION[a]

SOURCE OF EMISSION EXTRACT OR TYPE OF CONTROL	TRANSFORMATION		MUTATION	
	TYPE III FOCI	TF $(\times 10^{-5})$[b]	OUA^r COLONIES	MF $(\times 10^{-6})$[c]
CATERPILLAR DIESEL ENGINE	0	<0.68	1	0.18
OLDSMOBILE DIESEL ENGINE	1	0.33	2	0.53
ROOFING TAR	2	0.61	20	3.14[d]
NISSAN DIESEL ENGINE	14	3.43[d]	5	1.06[d]
COKE OVEN	6	2.13[d]	45	8.27[d]
MUSTANG II GASOLINE ENGINE	11	4.10[d]	27	4.49[d]
POSITIVE CONTROL (1 ug MNNG/ml)	29	12.34[d]	139	35.5[d]
NEGATIVE CONTROL (0.25% solvent)	0	<0.34	1	0.18

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[a] Doses for the various emission extracts were as discussed in Table 3, however, data from doses giving $<30\%$ survival were not included.

[b] TF = Transformation frequency for Type III foci only.

[c] MF = Mutation frequency.

[d] Data significantly different from combined negative controls at $p < 0.05$.

with each extract. These data were also combined to give an historic positive control for this assay series. Tables 4 and 5 list these resultant frequencies obtained from the combined data and compare each test article to the appropriate historic solvent control. It can be seen that in the absence of exogenous metabolic activation (Table 4) the Oldsmobile and Caterpillar diesel samples did not induce a significant number of mutants or transformed foci. However the Nissan, coke oven and Mustang II samples were quite active in inducing both a significant number of transformed foci and OUA^r colonies. The roofing tar pot sample induced a high frequency of OUA^r colonies but not a significant number of transformed foci.

When the same samples were tested in the presence of exogenous metabolic activation similar results were found (Table 5). The Caterpillar and Oldsmobile samples again showed no genotoxic activity in either the transformation or mutation assays. The roofing tar and Nissan diesel samples were mutagenic, but the frequencies of transformed foci which they induced were not statistically different from the combined negative controls. Both the coke oven and Mustang II samples induced a significantly high transformation and mutation frequency. Even though a large number of mutants were observed after treatment with the coke oven sample, the combination of the high spontaneous frequency which occurred during this specific assay and the lack of a dose response made the determination of significance difficult.

DISCUSSION

An assay system utilizing BALB/c 3T3 cells can function very well for routinely detecting both the transforming and mutagenic potential of many compounds. Historical data from our laboratory show remarkably low spontaneous transformation and mutation frequencies, allowing the system to detect chemicals with fairly weak biological activity. The simultaneous use of both the transformation and mutation endpoint greatly enhances the utility of the test system. The *in vitro* conditions that effect this assay system will be described at length elsewhere (manuscript in preparation).

Preliminary studies with MNNG and BaP, chemicals also used as positive controls in the absence (MNNG) and presence (BaP) of exogenous activation for each individual DCM extract assay, showed that the 3T3 cells can respond in a dose-dependent fashion for both mutagenesis and transformation (see Tables 1 and 2). However, when the complex

TABLE 5

TOTAL GENOTOXIC EVENTS OCCURING AFTER EXPOSURE TO VARIOUS DOSES OF EMISSION EXTRACTS IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION[a]

SOURCE OF EMISSION EXTRACT OR TYPE OF CONTROL	TRANSFORMATION		MUTATION	
	TYPE III FOCI	TF (x10^{-5})[b]	OUA^{r} COLONIES	MF (x10^{-6})[c]
CATERPILLAR DIESEL ENGINE	0	<0.30	1	0.20
OLDSMOBILE DIESEL ENGINE	0	<0.55	2	0.49
ROOFING TAR	4	1.07	11	1.73[d]
NISSAN DIESEL ENGINE	3	1.04	10	1.81[d]
COKE OVEN	6	2.41[d]	-	-[e]
MUSTANG II GASOLINE ENGINE	7	2.92[d]	19	3.97[d]
POSITIVE CONTROL (12.5 ug BaP/ml)	19	10.3[d]	48	14.2[d]
NEGATIVE CONTROL (0.25% solvent)	0	<0.36	1	0.26

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[a] Doses for the various emission extracts were as discussed in Table 3, however, data from doses giving <30% survival were not included.

[b] TF = Transformation frequency from Type III foci only.

[c] MF = Mutation frequency.

[d] Data significantly different from combined negative controls at $p < 0.05$.

[e] Spontaneous MF for this assay was significantly higher than the historical controls for this assay series.

emissions extracts were assayed, none yielded a dose-dependent increase in either mutation frequency or transformation frequency. This lack of dose response, even though individual doses of several of the extracts showed considerable genotoxic activity, could be due to a combination of reasons. The insoluble nature of the extracts in the aqueous growth media, the short (2 hours) exposure time, or the limited number of cells at risk, could all have been contributing factors.

The absence of a dose response makes quantitative comparisons among the extracts difficult. However, if the total number of genotoxic events (Type III foci or OUA^r clones) for the seven assay doses (five doses in two cases since only data obtained at survivals > 30% was used for these calculations) of each DCM extract tested was divided by the total number of cells at risk in that assay, a frequency could be obtained which was reflective of the activity of the extract over the range of concentrations assayed. The level of significance of each of these frequencies was determined by comparison to historic negative (solvent) control frequencies obtained by combining the individual negative controls from each DCM extract assay. Data from controls in the absence or presence of exogenous metabolic activation were combined separately. Using this type of analysis, Tables 4 and 5 show that DCM particulate extracts from the six sources can be rated as follows: Mustang II gasoline engine = coke oven > Nissan diesel engine > roofing tar. The Caterpillar and Oldsmobile diesel engine emission particulate extracts showed no significant genotoxic activity for 3T3 cells. However, when drawing any further conclusions, e.g., on the relative health hazards of the various emissions sources, the total amount of emission that each source normally emits to the atmosphere as well as the quantitative relationship of each extract to the actual emission (see Huisingh, et al., this symposium) must be considered.

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General Discussion

D. BRUSICK: Did you say that all of the samples were positive?

R. CURREN: I said we got a positive response in the form of type three foci with all the samples that we had. We would consider the Oldsmobile extract and the caterpillar extract moderately active. Benzopyrene, for example, tested in this system gives nothing.

W. THILLY: Many mutagenicity assays did show that benzopyrene whether in the alpha or the epsilon form, have activities within the same order of magnitude. For instance with the *Salmonella* typhimurium benzo(e)pyrene test,has about ten percent of the activity on a molar basis with benzo(a)pyrene.

R. CURREN: Actually I didn't show the slide, but we do see mutagenicity with benzo(e)pyrene.

W. THILLY: I think you might have been slightly misleading during your presentation when you said benzo(e)-pyrene has been shown negative in carcinogenicity assays and mutagenicity assays everywhere.

R. CURREN: I thought I said in general, low activity is found with benzo(e)pyrene. Of course, this activity could result from contamination with benzo(e)pyrene.

MUTAGENIC AND CARCINOGENIC POTENCY OF EXTRACTS OF DIESEL AND RELATED ENVIRONMENTAL EMISSIONS: TWO-STAGE CARCINOGENESIS IN SKIN TUMOR SENSITIVE MICE (SENCAR)[1]

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ABSTRACT

Skin tumors can be induced by the sequential application of a subthreshold dose of a carcinogen (initiation phase), followed by repetitive treatment with a noncarcinogenic tumor promoter. There is a very good dose-response relationship between the induction of the number of papillomas per mouse at early times (10 to 20 weeks) by either tumor initiators and promoters and the final carcinoma incidence after a longer latency (20 to 50 weeks) in SENCAR mice. This system not only can be used to determine the tumor initiating and promoting activities of a compound but if the agent is given repeatedly by itself one can also determine if it is a complete carcinogen, i.e., if it has both tumor initiating and promoting activity. In addition, if the agent is given concurrently with a known complete carcinogen or a tumor initiator one can also determine if the agent has co-carcinogenic or co-tumor initiating activity or even possibly anticarcinogenic activity. Likewise, if the agent is given concurrently with a known tumor promoter one can determine if the agent has co-promoting or anti-promoting activity. Using the

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SENCAR skin carcinogenesis system we have undertaken the determination of the skin carcinogenic, co-carcinogenic, tumor initiating and promoting activities of various diesel emission particle extracts as well as for comparative purposes, standards such as benzo(a)pyrene and 12-0-tetradecanoylphorbol-13-acetate and extracts of emissions from a gasoline engine, roofing tar, coke oven and cigarette smoke condensate. Most of the studies are still in progress but some preliminary data is available on the comparative tumor initiating activities of the various samples at 14 weeks. Caterpillar Diesel and cigarette smoke condensate were essentially without activity, whereas the Mustang gasoline Catalyst and Olds Diesel gave extremely low values although they were slightly above background. Roofing tar, coke oven and Nissan Diesel all gave moderate activity at high doses (1 to 10 mg) but when compared to benzo(a)pyrene were relatively low values.

INTRODUCTION

Based on knowledge derived from epidemiological studies, it is currently thought that the majority of cancers in humans are caused by environmental factors. Studies with experimental animals along with the epidemiological data have provided evidence that some chemicals in our environment are responsible for a significant proportion of such cancers.

Current information suggest that chemical carcinogenesis is a multistep process with one of the best studied models in this regard being the two-stage carcinogenesis system using mouse skin. Table 1 summarizes some aspects of carcinogenesis in experimental animals. Skin tumors can be induced by the sequential application of a subthreshold dose of a carcinogen (initiation phase) followed by repetitive treatment with a non-carcinogenic tumor promoter. The initiation phase requires only a single application of either a direct or indirect carcinogen at a subthreshold dose and is essentially irreversible, while the promotion phase is brought about by repetitive treatments after initiation and is initially reversible, later becoming irreversible. This system not only can be used to determine the tumor initiating and promoting activities of a compound but if the agent is given repeatedly by itself one can also determine if it is a complete carcinogen, i.e., if it has both tumor initiating and promoting activity. In addition, if the agent is given concurrently with a known complete car-

TABLE 1

CARCINOGENESIS IN MAN AND EXPERIMENTAL ANIMALS

1. Complete Carcinogenesis
2. Cocarcinogenesis
3. Tumor Initiation
4. Tumor Promotion
5. Additive and Synergistic Effects of Carcinogens, Tumor Initiators and Tumor Promoters
6. Co-initiating and Co-promoting Agents
7. Anti-Carcinogenesis
8. Anti-initiating and Anti-promoting Agents

cinogen or a tumor initiator one can also determine if the agent has co-carcinogenic or co-tumor initiating activity or even possibly anti-carcinogenic activity. Likewise, if the agent is given concurrently with a known tumor promoter one can determine if the agent has co-promoting or anti-promoting activity. Furthermore, like most carcinogenesis systems, skin carcinogens may have additive or synergistic effects. This system has provided an important model for studying carcinogenesis and for bioassaying carcinogenic agents.

Recently, the generality of the two-stage system or multistage carcinogenesis has been shown to exist in a number of systems besides the skin such as the liver, bladder, colon, esophagus, stomach, mammary, displacental as well as cells in culture (1). The various two-stage systems known and the initiating and promoting agents involved in each system are shown in Table 2. As is apparent, quite a diversity of initiating and promoting agents exists among the various two-stage systems.

Tumor Initiation

As stated earlier, the tumor initiation phase appears to be an irreversible step which probably involves a somatic cell mutation as evidenced by a good correlation between the carcinogenicity of many chemical carcinogens and their mutagenic activities (2, 3). Most tumor initiating agents either generate or are metabolically converted to electrophilic reactants, which bind covalently to cellular DNA and other macromolecules (4, 5). Previous studies have demonstrated a good correlation between the carcinogenicity of several polycyclic hydrocarbons and their ability to bind covalently to DNA (6, 7). The Millers have proposed a significant general theory to explain the initial event in chemical carcinogenesis which states that all chemical carcinogens that are not electrophilic reactants must be converted metabolically into a chemically reactive electrophilic form which then reacts with some critical macromolecule to initiate the carcinogenic process (4, 5).

Cocarcinogenesis and Tumor Promotion

The term cocarcinogenic action means the augmentation of carcinogenesis by a noncarcinogenic agent applied concomitantly with a carcinogen (8). A promoting agent is one applied repeatedly after a single dose of a tumor initiating agent that results in tumors (8). Tumor promoters can be either weak carcinogens or noncarcinogens. Some cocarcinogens such as croton oil, phorbol esters and anthralin are also tumor promoters. A cocarcinogen primarily has an influence on the initiation of the carcinogenic response through an effect on the carcinogen (permeability, metabolism or repairability of the carcinogenic damage) or the target tissue (for example, hormonal stimulation of mammary gland growth and croton oil stimulation of epidermal growth). A wide variety of agents have been found to be cocarcinogenic in experimental animals such as aliphatic hydrocarbons, aromatic hydrocarbons, aromatic hydrocarbons, phenols, long-chain acids, alcohols, hormones, radiation, viruses, food additives and diet, itself (1). Table 3 lists some of the more important cocarcinogenic agents that have been tested (1, 8). Due primarily to the ease of determining cocarcinogens as well as promoters in the skin system, most of the cocarcinogens have been discovered using that system.

Refer to Table 2 which summarizes the various two-stage

TABLE 2

TWO-STAGE CARCINOGENESIS SYSTEMS[a]

Organ System		
Mouse skin	Polycyclic aromatic hydrocarbons, urethane, direct-acting electrophiles [(epoxides, β-propiolactone, Bis chloromethylether, N-acetyoxyacetylaminofluorene and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)]	croton oil, phorbol esters, fatty acids and fatty acid esters, surface-active agents, linear long chain alkanes, tobacco smoke condensate and extracts of unburned tobacco, certain euphorbia macrocyclic diterpenes, citrus oil, anthralin and other phenols
Rat & mouse liver	2-acetamidofluorene,diethylnitrosamine, 2-methyl-N, N'-dimethyl-4-aminoazobenzene	phenobarbital, DDT; butylated hydroxytoluene, (BHT); polychlorobiphenyls,(PCB).
Mouse lung	Urethan, polycyclic aromatic hydrocarbons	BHT, phorbol
Rat colon	Dimethylhydrazine	Bile acids, high fat diet, high cholesterol diet
Rat bladder	N-methyl-N-nitrosourea	Saccharin, cyclamate
Rat & mouse mammary gland	Polycyclic aromatic	Hormones, high fat diet, phorbol
Rat stomach	N-methyl-N'-nitro-N-soguanidine	Surfactants
Rat esophagus	Diethylnitrosamine	Diet

TABLE 2 (continued)

Organ System		
Mouse cell culture systems	Polycyclic aromatic hydrocarbons, radiation	Phorbol esters, saccharin
Rat cell culture system	N-methyl-N'-nitro-N-nitrosoguanidine	phorbol esters

[a] See reference 1 for details

systems used and the various promoting agents associated with them. In many cases it is very difficult to distinguish between a cocarcinogen or a promoter because many of the agents have not been critically tested.

The phorbol ester tumor promoters are the most potent of the known tumor promoters, having been shown to have many cellular and biochemical effects (1,9,10). Some of the most important effects which seem to be intimately associated with the phorbol ester tumor promoters' action are as follows: (a) They induce changes in the phenotype of normal cells that mimic features of transformed cells. (b) They induce dedifferentiation of adult epidermal cells to embryonic looking cells. (c) They induce a 200-400 fold increase in epidermal ornithine decarboxylase activity which specifically correlates with tumor promotion. (d) They induce protease activity. (e) They stimulate phospholipid synthesis and DNA, RNA and protein synthesis. (f) They stimulate cellular proliferation. (g) They inhibit terminal differentiation not only in the skin but in other cellular systems such as Friend erythroleukemic cells, chicken myoblasts and neuroblastoma cells (1). Only a limited number of studies have been performed concerning the mechanism by which other tumor promoters act. Both phenobarbitol and BHT stimulate DNA synthesis followed by cell proliferation in the liver and lung, respectively (1).

TABLE 3

COCARCINOGENS IN VARIOUS TISSUES[a]

Skin

Catchol
pyrogallol
lauryl alcohol
decane
undecane
tetradecane
n-dodecane
pyrene
benzo(e)pyrene, B(e)P
fluoranthene
benzo(g, h,i)perylene
anthralin
croton oil
phorbol esters
phenols
nicotine
surfactants
radiation
viruses

Lung

asbestos
radiation
n-dodecane
ferric oxide
magnesium oxide
hypoxia

Mammary gland

hormones
estradiol
prolactin

Bladder

L-tryptophan
saccharin
cyclamate

Check Pouch

X-radiation
croton oil

Liver

cyclopropenoid fatty acids
alcohol

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[a] See reference 1 for details.

MATERIALS AND METHODS

Animals

SENCAR mice, a mouse stock selected for its increased sensitivity to carcinogenesis as described by Boutwell were used in this study (9). These mice were derived from breeding Charles River CD-1 mice with male STS Skin Tumor Sensitive originally derived from Rockland mice by Boutwell (9) and selecting for eight generations for sensitivity to DMBA-TPA two-stage system of tumorigenesis. These mice were initially obtained from Dr. Boutwell, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin, and now are being raised at the Oak Ridge National Laboratory, Oak Ridge, Tennessee. In addition, C57 black mice obtained from Jackson Laboratory, Jackson, Maine are also being used for these studies. Since the sensitivity to skin cancer is much less than the SENCAR mice this report will only deal with the results from the SENCAR mice.

Chemicals

TPA was obtained from Dr. P. Borchert, University of Minnesota, Minneapolis, MN. DMBA was purchased from Sigma Chemical Co., St. Louis, MO; BP and pyrene from Aldrich Chemical Co., Milwaukee, WI. The various diesel emission particle extracts as well as the other environmental emission samples were obtained from EPA under a code designation. All the agents were consistently prepared under yellow light immediately before use. In addition, all samples were applied topically in 0.2 ml of spectral quality acetone.

Tumor Experiments

The initial studies related to comparing tumor initiation and complete carcinogenesis of many carcinogens and the dose-response studies using DMBA, BP and TPA involved the use of only SENCAR mice with 30 to 40 females per group. The other studies related to the various emission samples obtained from EPA utilized two strains of mice (SENCAR and C57 black) with 80 mice per treatment group (40 males and 40 females). In addition, 5 dose levels were used for the carcinogenic, tumor initiating, cocarcinogenic and tumor promoting activities of the various samples. BP was used as the standard for the complete carcinogenesis and tumor initiation studies and TPA for the tumor promotion studies. Pyrene was used as the positive control for cocarcinogenesis studies with BP as the carcinogen. See Table 4 for a summary of objective and protocol. All the mice were shaved with surgical clippers 2 days before the initial treatment and only those mice in the resting phase of the hair cycle were used. Skin tumor formation was recorded weekly and papillomas greater than 2 mm in diameter were included in the cumulative total if they persisted for 1 week or longer. Both the number of mice with tumors and the number of tumors per mouse was determined and recorded weekly. At random, papillomas and carcinomas were removed for histological verification.

TABLE 4

OBJECTIVES OF THE DIESEL RESEARCH PROGRAM

Determine:	Complete carcinogenesis
	Cocarcinogenesis
	Tumor Initiation
	Tumor Promotion
Samples:	Olds Diesel
	Nissan Diesel
	U.W. Rabbit Diesel
	Caterpillar Diesel
	Mustang gasoline catalyst
	Coke Oven
	Roofing Tar
	Cigarette Smoke Condensate
Protocol:	SENCAR and C57 Black Mice
	40 males and 40 females
	5 dose levels
Standards:	Benzo(a)pyrene for complete carcinogenesis and tumor initiation
	Pyrene for cocarcinogenesis with benzo(a)-pyrene
	Phorbol ester (TPA) for tumor promotion

RESULTS AND DISCUSSION

Complete Carcinogenesis vs. Tumor Initiation

Whenever a known skin carcinogen has been appropriately tested, it has shown skin tumor initiating activity (9, 11-24). In a two-stage mouse skin system, initiation is the only stage that requires the presence of the carcinogen, and the measured carcinogenic potency of a chemical reflects its capacity for tumor initiation. The results in Table 5 show that there is both a good qualitative and quantitative correlation between the complete carcinogenic and tumor initiating activities of several chemical carcinogens in mouse skin. This is true when one considers the number of papillomas per mouse at early times (10 to 20 weeks) or the final carcinomas incidence after tumor initiation.

TABLE 5

Comparison of complete carcinogenesis and tumor initiation in mouse skin[a]

	Relative Potency[b]	
Compound	Complete Carcinogenesis (Carcinomas)	Tumor Initiation (Papillomas)
DMBA	100	100
MC	50	50
BP	30	30
2-OHBP	30	30
7BrMe-12MeBA	20	20
BP-7, 8-oxide	20	20
DB(a, h)A	20	20
BA	5 ± 5	5
DB(a, c)A	0	3
Pyrene	0	0
BP-4, 5-oxide	0	0
Anthracene	0	0

[a] This is a summary of over 100 compounds which shows that an excellent qualitative and quantitative correlation exists between complete carcinogenesis and tumor initiation in mouse skin.

[b] Relative potency was determined from dose-response data. DMBA was given a maximum value of 100.

It is possible that a carcinogen lacking promoting ability would not be detected when tested as a complete carcinogen. In this regard, however, we have found a number of chemical compounds that have tumor initiating activity but lack complete carcinogenic activity (12, 17-19, 22). These pure skin tumor initiators are listed in Table 6. Due to these considerations, we feel that it is important to test a compound as a tumor initiator as well as a complete carcinogen. The major disadvantage of the skin system which is also true of other carcinogenesis systems is that many carcinogens are tissue specific.

There is a good dose-response relationship of many carcinogens used as tumor initiators in the two-stage carcinogenesis system using SENCAR mice. This is illustrated in Table 7. A good dose response relationship exists for DMBA and BP to initiate skin tumors in SENCAR mice. As can be seen a good correlation exists between the number of papillomas per mouse at 15 weeks and the final carcinoma incidence at 50 weeks. The percent of mice with papillomas has also a reasonable correlation but the dose response is very narrow. Table 8 shows the dose-response ability of TPA to promote tumors after DMBA initiation. As was the case for tumor initiation, there is also a very good dose-response relationship for tumor promotion when considering either the number of papillomas per mouse at 15 weeks or the percent of mice with squamous cell carcinomas at 50 weeks. Similar results have been reported using Charles River CD-1 mice (13, 16) or ICR/Ha Swiss mice (25, 26).

TABLE 6

Agents that are possibly pure tumor initiators

Skin Tumor Initiators	Relative[a] Potency
BP-7, 8-diol-9, 10-epoxide	25
MNNG	15
BA-3, 4-diol-1, 2-epoxide	2
BA	0.5
DB(a, c)A	0.2
Chrysene	0.1
Urethan	0.1

[a] Relative potency was determined from dose-response data. DMBA was given a maximum value of 100.

TABLE 7

DOSE RESPONSE STUDIES ON THE ABILITY OF DMBA AND BP TO INITIATE SKIN TUMORS IN SENCAR MICE

The mice were treated one week after initiation with twice weekly applications of 5 ug of TPA

Initiator	Dose (nmoles)	# of papillomas per mouse at 15 weeks	% of mice with papillomas at 15 weeks	% of mice with carcinomas at 50 weeks
DMBA	100	22	100	100
DMBA	10	6.8	100	40
DMBA	1	3.2	93	22
DMBA	0.1	0.5	20	5
BP	200	7.5	100	55
BP	100	3.2	78	30
BP	50	1.4	60	18

TABLE 8

DOSE-RESPONSE STUDIES ON THE ABILITY OF TPA TO PROMOTE TUMORS AFTER DMBA INITIATION

The mice were initiated with 10 nmoles of DMBA and promoted one week later with various dose levels of TPA

Promoter	dose (ug)	time to first papilloma (wks)	# of papillomas per mouse at 15 weeks	% of mice with papillomas at 15 weeks	% of mice with carcinomas at 15 weeks
TPA	10	8	3.0	100	32
TPA	5	6	7.2	100	46
TPA	2	7	6.5	100	45
TPA	1	8	3.6	80	25
TPA	0.1	11	0.4	5	8

Studies on the Carcinogenesis of Diesel Emission Particle Extracts

We were interested in determining the skin carcinogenic, cocarcinogenic, tumor initiating and promoting activities of diesel emission particle extracts. In addition, we wanted to compare the above results to other environmental emission particle extracts (coke oven, roofing tar, cigarette smoke and gasoline engine), as well as positive controls such as BP for tumor initiation and complete carcinogenesis, TPA for promotion and pyrene for cocarcinogenesis with BP. Presently, we can only present a preliminary comparative study on the skin tumor initiating activities of all the samples. Figure 1 shows that an excellent dose-response relationship exists for the initiating ability of BP at 14 weeks. A similar dose-response relationship exists for the tumor initiating activity of the Nissan Diesel sample but using mg quantities for initiation (Figure 2). The coke oven sample also gave a reasonable dose-response curve (Figure 3). Table 9 summarizes the skin tumor initiating activities of the various samples. The comparison of their tumor initiating activities was made at 14 weeks. The tumor response is expressed as the mean number of papillomas per mouse per mg. The values represent the slope from a linear regression analysis when plotting data from dose-response studies. Caterpillar Diesel and cigarette smoke condensate were for all practical purposes zero whereas Mustang gasoline and Olds Diesel gave extremely low values, although they were above background. Roofing tar, Nissan Diesel and coke oven all gave moderate activity at high doses (1 to 10 mg) but when compared to benzo(a)pyrene were relatively low values. 10 Milligrams of the roofing tar sample had a tumor initiating activity comparable to 50 μg of BP, whereas 10 mg of the Nissan Diesel was comparable to 60 μg of BP, and 10 mg of the coke oven sample was comparable to 80 μg of BP.

TABLE 9

Comparative Skin Tumor Initiating Activities of Extracts of Diesel and Related Environmental Emissions[a]

Sample	# Papillomas/mouse/mg[b] (14 weeks)	R^2
Caterpillar Diesel	0	
Nissan Diesel	0.258	0.996
Olds Diesel	0.115	0.95
Mustang Gasoline	0.09	--
Cigarette Smoke Condensate	0	--
Roofing Tar	0.182	0.999
Coke Oven Emissions	0.307	0.876
Benzo(a)pyrene	46.2	0.984

[a] 40 males and 40 females were initiated with the various samples and promoted one week later by twice weekly applications of 2 µg of TPA.

[b] The values represent the slope from the linear regression analysis of the dose-response studies and measure of fit (R^2).

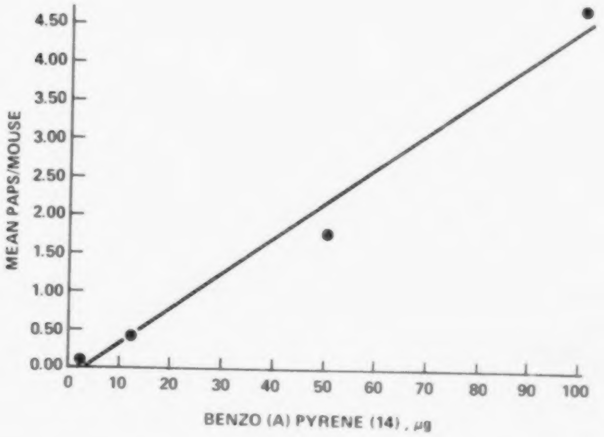

Figure 1. Dose-response relationship for BP applied topically to male and female SENCAR mice once and followed 1 week later by twice weekly application of 2 µg of TPA for 14 weeks.

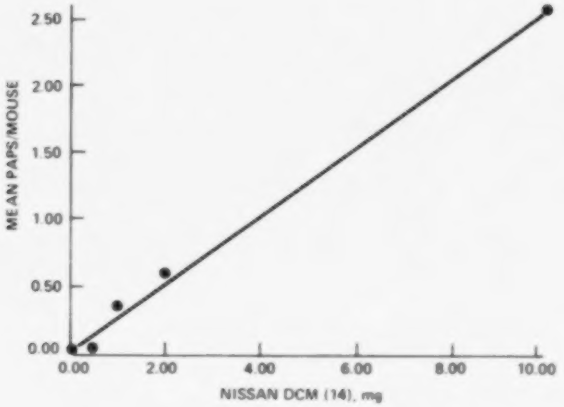

Figure 2. Dose-response relationship for the Nissan Diesel sample (Nissan DCM) applied topically to male and female SENCAR mice and followed 1 week later by twice weekly applications of 2 μg of TPA for 14 weeks.

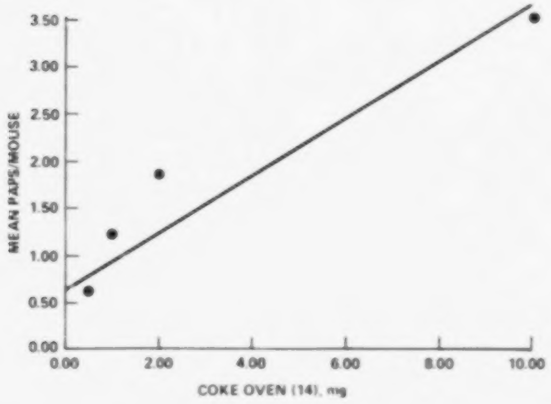

Figure 3. Dose-response relationship for the coke oven sample applied topically to male and female SENCAR mice and followed 1 week later by twice weekly applications of 2 μg of TPA for 14 weeks.

Abbreviations used: TPA, 12-0-tetradecanoylphorbol-13-acetate; DMBA, 7,12-dimethylbenz(a)anthracene; BP, benzo(a)pyrene; MC, 3-methylcholanthrene; 2-OHBP, 2-hydroxybenzo(a)pyrene; 7BrMe-12MeBA, 7-Bromomethyl-12-methylbenz(a)anthracene; BP-7, 8-oxide, benzo(a)pyrene-7, 8-oxide; DB(a, h)A, dibenz(a, h)anthracene; BA, benz(a)anthracene; DB(a, c)A, dibenz(a, c)anthracene; BP-4, 5-oxide, benzo(a)pyrene 4, 5-oxide; BP-7, 8-diol-9, 10-epoxide, (±)-7β, 8α-dihydroxy-9α-10α-epoxy-7, 8, 9, 10-tetrahydrobenzo(a)pyrene; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; BA-3, 4-diol-1, 2-epoxide; (±)-3α, 4β-dihydroxy-1α, 2α-epoxy-1, 2, 3, 4-tetrahydrobenz(a)anthracene.

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General Discussion

R. ALBERT: Could you discuss the negative result of cigarette condensate which is kind of peculiar since cigarette condensate certainly has been shown to be a complete skin carcinogen. I wonder whether or not, for example, you use enough of it so that the benzopyrene content of the cigarette tar should have produced a response.

T. SLAGA: The maximum dose level that we used was ten milligrams. The maximum we could get into solution and put on the skin was two milligrams at a time. we used five applications of 2 mg each to get our maximum dose of ten milligrams. The data that has already been published in terms of cigarette smoke condensate by Hoffmann's group and a few others were at higher levels than we are dealing with. I think that at increased concentrations, we would achieve tumor initiation. These are short exposures too, only 14 weeks. It is possible that the latency period may be much longer for the cigarette smoke condensate. The tumor initiation studies as well as the complete carcinogenesis studies are planned to last at least 60 weeks. So then, these results are preliminary and might change as the experiments progresses.

SPEAKER: Tom, most of us feel that the skin carcinogenesis assay, is one of the best, if not the best and most quantitative in vivo carcinogenesis assay that we have at the present time. I wonder whether you could address the question of what type of carcinogen we can hope to pick up with the skin carcinogenesis assay. I think that is becoming an extremely crucial question since in complex mixtures, such as diesel exhaust, we are obviously dealing with a great variety of carcinogens and initiators. It becomes very essential for us to know what we can expect to pick up with that assay and what kinds of compounds we might miss with that assay.

T. SLAGA: I tried to emphasize in the beginning of the talk that there is tissue specificity to a large number of carcinogens, but in another slide I tried to point out a number of the various different agents that are positie when tested on the skin. These include not only the polycyclic hydrocarbons but a number of alkylatic agents, urethane,etc. It probably picks up the widest range of carcinogens as any in vivo system that I know. In general, most in vivo systems are a lot more specific then skin. A disadvantage of the skin system tht we are presently utilizing is that the skin does not always metabolize every procarcinogen to the active metabolite. We are modifying the system by injecting the test substance. IP, in order to allow the liver or any other tissue to metabolize it. We have some preliminary data with hydrocarbons that we can inject them IP, promote on the skin, and get a nice tumor response. We have some data with acetoaminoflurene which is a liver carcinogen showing that the skin doesn't appear to metabolize it. If you give it repetitively to the skin you will not get skin cancer. If you give it to IP, at a reasonable dose, then follow topically with a tumor promoter, then you get skin cancer. This may be true for a number of other agents which we are pursuing right now.

R. BILES: Can you tell me the number of animals you used per group and also the number of animals with papillomas. Was your data presented as the numbers of mean papillomas per animal.

T. SLAGA: I tried to give some idea on a couple of the dose response studies that I presented, showing the number of papillomas per mouse and how that correlates to the percent of animals with tumors which further correlates with the final carcinoma yield.

R. BILES: What about animals with papillomas? How many animals per group did you use?

T. SLAGA: Eighty. On one of my slides I showed that we used 40 males and 40 females per dose. In the last couple of slides, males and females were averaged together since there was essentially no difference. In general once we reach a certain level of number of benign tumors per mmouse, all of our animals get papillomas. The dose response relationship when presented on the percent of animals with tumors, only goes from a zero to 100 percent. Therefore, once all of your animals have tumors, be it only one tumor or ten tumors, you get the same type of results. This results in a very narrow dose response relationship. If you look at the number of papillomas per mouse, then you extend that dose response relationship.

R. BILES: So all of your animals had papillomas.

T. SLAGA: At low doses there may be fewer papillomas per animal but all of them had papillomas.

D. HOFFMANN: I do differ from you based on literature reports which have very clearly shown when combustion products, tobacco tar, gasoline engine exhaust are fractionated, mutation activity is observed only in the PAH fraction. Since we know from all the mutagenicity studies that the major activity is not in the PAH fraction. I doubt that you can pick up the carcinogenicity of those agents which are carcinogenic but not mutagenic. There is no evidence that neutral components, in combustion products which are not PAH produce tumors.

T. SLAGA: Your question is a little misleading. When I stated that you get a tumor response with those various mixtures, I wans't trying to say that it was related to the hydrocarbons though I gave it in terms of PAH equivalent response. The fact that some of our data has come out negative, for tumor initiation in these relatively sensitive animals, could be related to a dose relationship. In which we are not using a high enough dose. When you are dealing with a complex mixture, and are looking at the whole mixture, you have a number of factors that not only could cause initiation of tumors, but you have a number of agents that could also negate that response. I think your studies showed that by fractionation one can get the specific fractions that possess more tumor initiating and promoting, actiity.

L. DEPASS: Have you seen any kind of dose response relationship in terms of time to papilloma in your study?

T. SLAGA: We haven't really analyzed our data in terms of which compound or mixture gave rise to tumors the soonest. We have that data but I haven't really tried to rank them according to time to tumor data. Basically I would say that the samples that are responsive the nissan, the coke oven, and the roofing tar, all had about the same latency which is very close to what benz(a)pyrene had. However, the number of tumors was much less compared to benz(a)pyrene.

MUTAGENIC AND CARCINOGENIC POTENCY OF EXTRACTS OF DIESEL AND RELATED ENVIRONMENTAL EMISSIONS: SUMMARY AND DISCUSSION OF THE RESULTS

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ABSTRACT

The proposed conversion from gasoline powered automobiles to diesel powered vehicles has prompted the Environmental Protection Agency to evaluate the potential health effects associated with exposure to diesel emissions. At present, there is no direct epidemiological link between this exposure and human health. Therefore, a research program was constructed to compare the health effects associated with diesel emissions with those from other emission sources for which epidemiological information was available. The emission sources chosen were cigarette smoke, roofing tar, and coke oven. An additional comparative emission source which was evaluated was a gasoline catalyst engine.

Respirable particles from a variety of combustion sources have the potential of being carcinogenic and mutagenic. The objective of these studies was to determine the relative biological activity of the organic material adsorbed on these particles in both *in vitro* mutagenesis and *in*

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vitro and *in vivo* carcinogenesis bioassays. The organic extracts from the following series of emission sources were quantitatively bioassayed in a matrix of tests for their carcinogenic and mutagenic activity: (1) a light-duty Oldsmobile diesel 350 engine; (2) a heavy-duty Caterpillar diesel engine; (3) a light-duty Nissan engine; (4) a Volkswagen Rabbit diesel engine; (5) cigarette smoke; (6) roofing tar; (7) coke oven; and (8) a gasoline catalyst Mustang.

The test matrix consisted of the following bioassays: reverse mutation in *Salmonella typhimurium*; mitotic recombination in *Saccharomyces cerevisiae*; DNA damage in Syrian hamster embryo cells (SHE); sister chromatid exchange in CHO cells; gene mutation in L5178Y mouse lymphoma cells, Balb/c 3T3 mouse embryo fibroblasts and CHO cells; viral enhancement of SHE cells; oncogenic transformation in Balb/c 3T3 cells; and skin tumor initiation in Sencar and C57 black mice.

The results of this test matrix are discussed.

INTRODUCTION

The proposed conversion from gasoline powered automobiles to diesel powered vehicles has prompted the Environmental Protection Agency to evaluate the potential health effects associated with exposure to diesel emissions (1). At present, there is no direct epidemiological link between this exposure and human health (2). Therefore, a research program was constructed to compare the health effects associated with diesel emissions with those from other emission sources for which epidemiological information was available. The emission sources chosen were cigarette smoke, roofing tar, and coke oven. An additional comparative emission source which was evaluated was a gasoline catalyst vehicle.

Respirable particles from a variety of combustion sources have the potential of being carcinogenic and mutagenic (3). The objective of these studies was to determine the relative biological activity of the organic material adsorbed on these particles in both *in vitro* and *in vivo* mutagenesis and carcinogenesis bioassays. The organic extracts from the following series of emission sources were quantitatively bioassayed in a matrix of tests for their carcinogenic and mutagenic activity: (1) a light-duty Oldsmobile diesel 350 engine, (2) a heavy-duty Caterpillar 3304 diesel engine, (3) a light-duty Datsun Nissan 220C engine, (4) a Volkswagen turbocharged Rabbit diesel engine, (5) cigarette smoke, (6) roofing tar, (7) coke oven, and (8) an unleaded gasoline catalyst Mustang II.

The collection, characterization, and description of these samples are described by Huisingh *et al*. (4).

The test matrix consisted of the following bioassays: reverse mutation in *Salmonella typhimurium*; mitotic recombination in *Saccharomyces cerevisiae*; DNA breakage in Syrian hamster embryo cells (SHE); sister chromatid exchange in Chinese hamster ovary (CHO) cells; gene mutation in L5178Y mouse lymphoma cells, Balb/c 3T3 mouse embryo fibroblasts, and CHO cells; viral enhancement in SHE cells; oncogenic transformation in Balb/c 3T3 cells; and skin tumor initiation in Sencar and C57 black mice.

The potency of complex mixtures of organic compounds in biological systems will depend on the uptake, distribution, metabolism, and binding of each component of the mixture in each biological system; the specific biological endpoint (e.g., gene mutation); the sensitivity of each biological system to the individual components of the mixture; the possible interactions, both synergistic and antagonistic, which arise from the exposure to multiple agents; and the type of quantitative method which is applied to the data. Differences in these parameters will result in altered potency.

Aware of these problems and shortcomings, we have attempted to correlate biological end effects from a variety of carcinogenesis and mutagenesis bioassays with the nature or source of a series of combustion samples. The purpose of this comparative study is to evaluate each of the *in vitro* bioassay systems in comparison with each other and with the *in vivo* system. This paper will summarize and discuss the results of the bioassays reported in the session on the Mutagenic and Carcinogenic Potency of Extracts of Diesel and Related Environmental Emissions (5-9).

RESULTS AND DISCUSSION

QUANTITATION METHOD

General Procedures

The method for relating effect with dose depends on the bioassay being considered. The bacterial mutation system of Ames (AMES), the sister chromatid exchange bioassay in Chinese hamster ovary cells (SCE), the mammalian cell mutagenesis bioassay in L5178Y mouse lymphoma cells, (L5178Y) and the mouse skin tumor initiation bioassay (TUMOR INITIAT) gave good dose-response relationships and the slope of the linear portion of the dose response curve was chosen for potency estimation (Table 1). The Balb/c

TABLE 1. COMPARATIVE POTENCY: QUANTITATION METHOD

	MUTAGENESIS				CARCINOGENESIS		
BIOASSAYS:	AMES	SCE	L5178Y	BALB	VIRAL ENHANCE	BALB	TUMOR INITIAT
QUANTITATION METHOD:	Slope	Slope	Slope	LECT	LECT	LECT	Slope

LECT = Lowest effective concentration tested.
Slope = Slope of the linear portion of the dose response curve.

3T3 mutagenesis and oncogenic transformation bioassays (BALB) did not give good dose response with the samples tested and doses chosen. A quantitation method was therefore applied which utilized the lowest effective concentration tested (LECT) which exerted a biological response. It is to be emphasized, however, that with pure agents and more closely spaced doses the Balb assay does respond in a dose-related fashion. The viral enhancement bioassay in Syrian hamster embryo cells (VIRAL ENHANCE) gave dose related response information in terms of absolute frequency but for comparison to the Balb system and due to the kind of statistics applied to the assay, the LECT method was chosen. It is possible that the two methods, slope and LECT, when applied to the same data could give dissimilar relative rankings. Bioassay results in Saccharomyces cerevisiae, DNA damage in SHE cells, gene mutation in CHO cells, and skin tumor initiation in C57 black mice were not utilized in this analysis due to marginal results.

Normalization of Data

In order to reduce or normalize the potencies in the bioassays to a common denominator, the following system was applied: the activity of the Nissan diesel sample regardless of bioassay type or quantitation method used was given a value of 100. All the results of other emission samples were then related to the Nissan sample. For example, in the Ames bioassay, Strain TA-98 less metabolic activation, the potency of Nissan and Oldsmobile by the slope method was 1225 and 615 revertants/100 µg, respectively. Assuming the Nissan potency equals 100 by simple mathematical relationship, the Oldsmobile sample equals 50 or one-half the potency of the Nissan sample. In the case of a LECT potency, the inverse relationship is applied. For example if the LECT for Nissan is 75 µg/ml and that for CAT is 300 μg/ml, assuming Nissan equals 100, then CAT equals 75/300 x 100 or 25, one-quarter the potency of the Nissan sample. Using this kind of analysis, the absolute data was converted to normalized values. The reason the Nissan was chosen as the sample to normalize to was that it was the only diesel sample tested in all bioassays which gave positive results.

COMPARATIVE RANKINGS

Gene Mutation Assays

A comparison of the test results of the eight samples plus the positive control in the three gene mutation assays is found in Table 2. The three gene mutation assays compared

TABLE 2. COMPARATIVE RANKINGS: GENE MUTATION ASSAYS

		AMES[a]		L-5178Y[b]		BALB/3T3[c]	
SAMPLE		-MA	+MA[d]	-MA	+MA	-MA	+MA
DIESEL:	Cat	5.4	4.3	16	I[e]	75	25
	Nissan	100	100	100	100	100	100
	Olds	50	23	58	64	94	58
	VW Rab.	33	22	21	50	I	I
GASOLINE:	Mustang	11	25	32	36	300	7500
COMPARATIVE SOURCES:	Cigarette	0	7	42	21	300	300
	Coke	13	18	26	339	300	15
	Roof Tar	0	7	16	850	150	7500
STANDARDS:							
	B(a)P	0	1112	0	189		25000
	MNNG					25000	

[a] Salmonella typhimurium strain TA-98.

[b] L-5178Y mouse lymphoma cells (TK$^{+/-}$ locus).

[c] BALB 3T3 mouse embryo fibroblasts (ATPase locus).

[d] Metabolic activation by an Aroclor-1254 induced rat hepatic S-9.

[e] Testing incomplete at this time.

were Ames strain TA-98, L5178Y at the $TK^{+/-}$ locus, and BALB 3T3 at the ATPase locus. Each system was performed without (-MA) and with (+MA) metabolic activation which consisted of an Aroclor-1254 induced rat hepatic S-9. The emission samples were previously described and are abbreviated as Cat for heavy duty Caterpillar; Nissan; Olds for Oldsmobile 350; VW Rabbit for the turbocharged diesel Rabbit; Mustang for the unleaded catalyst gasoline engine; Cigarette for standard cigarette smoke condensate; Coke for coke oven emissions; Roof Tar for roofing tar emissions; B(a)P for benzo(a)pyrene; and MNNG for N-methyl-N'-nitro-N-nitrosoguanidine.

The Nissan, as per our definition, has a value of 100 in all assays. Both the Ames and lymphoma assay (without activation) show the same relative potency within the diesel samples with Nissan > Olds > Rabbit > Cat. When the gasoline sample is included, the rankings are: Ames: Nissan > Olds > Rabbit > Mustang > Cat; mouse lymphoma: Nissan > Olds > Mustang > Rabbit > Cat. Both of these cell types lack the oxidative enzymes required for the activation of mutagens or carcinogens. The diesel and gasoline samples show primarily direct acting activity. Balb 3T3 cells possess the enzymes required for the activation of carcinogens especially polycyclic aromatics (8) and therefore one cannot compare all these systems across the board and expect similar results. Comparison of the three systems with metabolic activation showed the relative ranking (with some exceptions) to be: Nissan > Olds > Rabbit > Cat.

The comparative sources samples generally show more metabolic activation dependence for maximal effect and less quantitative correlation in the gene mutation assays.

DNA Damage Assay

The mobile source samples tested for sister chromatid exchange in CHO cells, without activation, (Table 3) gave the following ranking: Nissan > Rabbit, Mustang >> Cat, Olds.

In the presence of metabolic activation, the SCE results with the comparative sources ranked in similar order with the mouse lymphoma results: Roof Tar > Coke > Cigarette.

TABLE 3. COMPARATIVE RANKINGS: DNA DAMAGE ASSAY

	SAMPLE	SCE (CHO)[a] -MA	SCE (CHO)[a] +MA[b]
DIESEL:	Cat	4	0
	Nissan	100	100
	Olds	0	0
	VW Rab	30	50
GASOLINE:	Mustang	29	I[c]
COMPARATIVE SOURCES:	Cigarette	47	0
	Coke	171	44
	Roof Tar	60	291
STANDARDS:	B(a)P	0	1750

[a] Sister chromatid exchange in Chinese hamster ovary cells.

[b] Metabolic activation by an Aroclor-1254 induced rat hepatic S-9.

[c] Testing incomplete at this time.

Oncogenic Transformation

The results from assays which relate to the transformation of cells in culture are found in Table 4. Both of these systems were evaluated by the LECT method. The viral enhancement assay measures the ability of a chemical to enhance viral transformation in mammalian cells while the BALB assay measures the ability of the chemical to directly transform mammalian cells. SHE cells like BALB cells contain the enzymes needed for the metabolic activiation

TABLE 4. COMPARATIVE RANKINGS: ONCOGENIC TRANSFORMATION ASSAYS

SAMPLE		VIRAL ENHANCEMENT (SHE)	BALB 3T3 -MA	BALB 3T3 +MA[a]
DIESEL:	Cat	0	1.3	0
	Nissan	100	100	100
	Olds	0	1.9	0
	VW Rab	50	NT[b]	NT
GASOLINE:	Mustang	50	100	2000
COMPARATIVE SOURCES:	Cigarette	200	I[c]	I
	Coke	800	10	I
	Roof Tar	1040	I	500
STANDARDS:	B(a)P	50000		16700
	MNNG	31250	8300	

[a] Metabolic activation by an Aroclor-1254 induced rat hepatic S-9

[b] Not tested.

[c] Testing incomplete at this time.

of carcinogens especially polycyclic aromatics (8). At this time not all the data points are complete in the Balb 3T3 assay. The mobile source samples, presently rank for viral enhancement in the absence of metabolic activation: Nissan > Rabbit > Mustang >> Cat, Olds, while the comparative samples rank: Roof Tar > Coke > Cigarette.

Mouse Skin Tumorigenesis

The results obtained in the Sencar mouse skin tumorigenesis experiments for tumor initiators have been compared at 14 weeks after treatment (interim score) for all the samples and at 27 weeks of treatment (final score) for three samples (Table 5). The interim score is represented here in both absolute terms (papillomas/mouse/mg) and in the

TABLE 5. COMPARATIVE RANKINGS: SENCAR MOUSE SKIN TUMORIGENESIS

		TUMOR INITIATION		
		INTERIM SCORE[a]		FINAL SCORE
	SAMPLE	RANKING	POTENCY[b]	POTENCY[b]
DIESEL:	Cat	0	0.00	0.00
	Nissan	100	0.258	
	Olds	45	0.115	0.145
	VW Rab	I[c]	I	
GASOLINE:	Mustang	35	0.090	
COMPARATIVE SOURCES:	Cigarette	0	0.00	
	Coke	119	0.307	
	Roof Tar	71	0.182	
STANDARDS:	B(a)P	17900	46.2	71.6

[a] Interim score 14 weeks after treatment.

[b] Papillomas/mouse/mg.

[c] Testing incomplete at this time.

normalized rankings while the final scoring for comparison is in absolute terms. There are, as expected, differences in the slope potencies from incomplete vs completed experiments but those differences do not exceed 2-fold in the Olds and B(a)P samples. The Cat and Cigarette samples were negative in this assay system up to 10,000 μg/mouse. The lack of activity of the cigarette smoke condensate may be due in part to the method of sample collection. This sample is not an organic extract of particles as are the other samples and is therefore comparatively much less concentrated. This may explain the very low percentage of benzo(a)pyrene found in the cigarette smoke condensate sample (Table 6). The Olds, Mustang, and Roofing Tar were less than Nissan while the Coke oven sample was slightly higher. The ranking of the mobile source samples was: Nissan > Olds > Mustang >> Cat.

TABLE 6. CORRELATION BETWEEN ORGANICS AND TUMOR INITIATION

		PERCENT ORGANIC EXTRACTABLE	ng B(a)P/ mg EXT	MOUSE SKIN TUMOR INITIATION[a]
DIESEL:	Cat	27	2	0.00
	Nissan	8	1173	0.258
	Olds	17	2	0.115
	VW Rab	18	26	I[b]
GASOLINE:	Mustang	43	103	0.09
COMPARATIVE SOURCES:	Cigarette	-	< 1	0.00
	Coke	7	478	0.307
	Roof Tar	> 99	889	0.182
STANDARDS:	B(a)P	-	10^6	46.2

[a] Interim score 14 weeks after treatment (papillomas/mouse/mg).

[b] Testing incomplete at this time.

A comparison of the amount of B(a)P per milligram extract and the potencies of those samples as skin tumor initiators is found in Table 6. There is a semiquantitative correlation observed, except for the Olds sample, between ng B(a)P/mg extract and papillomas/mouse/mg extract.

COMPARISON ACROSS TEST SYSTEMS

The relative rankings of the mobile source samples are listed in Table 7. These results are from those bioassays performed in the presence of exogenous metabolic activation.

There is a consistency in these results with the Nissan sample the most potent and the Cat sample the least potent in all bioassays.

The relative rankings of the comparative source samples is found in Table 8. In three of five systems, the identical rank order of Roof Tar > Coke > Cigarette is found, and in all five systems, the Cigarette sample was the least potent.

TABLE 7. RELATIVE RANKINGS OF MOBILE SOURCE SAMPLES

BIOASSAY	RANK ORDER
AMES[a]	Nissan > Mustang, Olds, Rabbit >> Cat
L5178Y[a]	Nissan > Olds > Rabbit > Mustang
SCE[a]	Nissan > Rabbit >> Cat, Olds
VIRAL ENHANCEMENT	Nissan > Mustang, Rabbit >> Cat, Olds
TUMOR INITIATION[b]	Nissan > Olds > Mustang >> Cat

[a] In the presence of an Aroclor-1254 induced rat hepatic S-9

[b] Mouse skin tumor initiation in Sencar mice 14 weeks after treatment

TABLE 8. RELATIVE RANKINGS OF COMPARATIVE SOURCE SAMPLES

BIOASSAY	RANK ORDER
AMES[a]	Coke > Roof Tar, Cigarette
L5178Y[a]	Roof Tar > Coke > Cigarette
SCE[a]	Roof Tar > Coke >> Cigarette
VIRAL ENHANCEMENT	Roof Tar > Coke > Cigarette
TUMOR INITIATION[b]	Coke > Roof Tar >> Cigarette

[a] In the presence of an Aroclor-1254 induced rat hepatic S-9

[b] Mouse skin tumor initiation in Sencar mice 14 weeks after treatment

TABLE 9. COMPARATIVE POTENCY RANKINGS

	MUTAGENESIS				CARCINOGENESIS		
	AMES[a]	SCE[a]	L-5178Y[a]	BALB[a]	VIRAL ENHANCE	BALB[a]	TUMOR INITIATION[b]
DIESEL: Cat	4.3	0	I[c]	25	0	0	0
Nissan	100	100	100	100	100	100	100
Olds	23	0	64	58	0	0	45
VW Rab	22	50	50	NT[d]	50	NT	I
GASOLINE:							
Mustang	25	I	36	7500	50	2000	35
COMPARATIVE SOURCES:							
Cigarette	7	0	21	300	200	I	0
Coke	18	44	339	15	800	I	119
Roof Tar	7	291	850	7500	1040	500	71
STANDARDS:							
B(a)P	1112	1750	189	25000	50000	16700	17900

[a] In the presence of an Aroclor-1254 induced rat hepatic S-9.
[b] Mouse skin tumor initiation in Sencar mice after 14 weeks of treatment.
[c] Testing incomplete at this time.
[d] Not tested.

The normalized rankings for 7 bioassays are compared in Table 9. The results presented are from those bioassays performed in the presence of exogenous metabolic activation including Ames, SCE, L5178Y and Balb-mutagenesis and transformation. The quantitative results from the mobile source samples show a general overall consistency. Cat, a very weak sample in Ames is inactive in SCE, viral enhancement, Balb transformation, and mouse skin tumor initiation. Olds, weak in Ames, L5178Y, Balb mutation, and mouse skin tumor initiation is inactive in SCE, viral enhancement, and BALB transformation. In three of the four assays for which test data is available, SCE, L5178Y, and viral enhancement, the Rabbit sample gave almost identical quantitative results. The Mustang sample gave similar results in Ames, L5178Y, and mouse skin tumor initiation, while markedly dissimilar results were obtained in the Balb assay.

With the comparative source samples, there is little agreement between the quantitative results from these bioassays with the exception of the Roofing Tar sample in L5178Y, viral enhancement and Balb transformation.

In theory, gene mutation and skin tumor initiation arise from similar one hit, single process, irreversible mechanisms and should give similar results assuming equal toxicity and mutagen/carcinogen transport/activation by the various cell types. A comparison of the results of the mobile source samples in Ames and L5178Y gene mutation with those results in mouse skin tumor initiation seem to support this hypothesis.

In conclusion, a series of extracts from diesel and gasoline emission samples were evaluated in a battery of bioassays and a broad general agreement was found among most of the bioassays. Similar experimentation with the Cigarette, Coke oven, and Roofing Tar samples produced dissimilar results. Additional experimentation and analysis will continue in this important area of environmental mutagenesis and carcinogenesis.

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General Discussion

SPEAKER: As the data stands now, number one; what is the bottom line in terms of mutagenesis; and number two; in terms of carcinogenesis?

S. NESNOW: I think as the data stands now, we have evidence that certain of these samples do not exhibit any activity. Then there are a range of samples that exert activity which are fairly comparable with the Nissan and there are other samples, that are very high. I think the bottom line is that given a multitude of these systems we have confirmed again and again the carcinogenicity and mutagenicity of most of these samples. We are sort of breaking new ground trying to test potencies and trying to evaluate and compare samples.

Session VI

MUTAGENICITY OF INHALED DIESEL EMISSIONS

Chairman:

Dr. David Brusick

A Study of Diesel Emissions on Drosophila.
Schuler, Ronald L. and Richard W. Niemeier.

Metaphase Analysis, Micronuclei Assay and Urinary Mutagenicity Assay of Mice Exposed to Diesel Emissions.
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A STUDY OF DIESEL EMISSIONS ON DROSOPHILA

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ABSTRACT

A sex-linked recessive lethal test was performed on male fruit flies of the species Drosophila melanogaster, (Oregon-R strain), exposed to an approximate five-fold dilution of exhaust from a diesel engine. The eight hour exposure was achieved by drawing diluted diesel exhaust from a three cubic meter stainless steel exposure chamber housing laboratory animals through a two liter reaction flask modified for use with Drosophila. A preconditioned sampling bag was used to collect the emissions after passing through the exposure chamber containing the flies. Results of analyses performed on the diesel exhaust mixture showed: carbon dioxide - 0.17%, carbon monoxide - 12.2 ppm, hydrocarbons - 11.6 ppm, nitrogen oxide - 3.8 ppm, nitrogen dioxide - 2.9 ppm, sulphur dioxide - 1.0 ppm, and particulates - 2.18 mg/m^3.

Two broods of the F_2 generation were investigated for the occurrence of recessive lethal events. These broods approximated the developing gametogenic stages of mature sperm (P_1 matings on days 2 and 3 post-exposure) and spermatocytes (P_1 matings on days 8 and 9). Additionally, the F_3 generation was evaluated for the occurrence of mosaic recessive lethal events which might escape detection in the F_2 generation. An equal number of F_2 and F_3 flies for both broods served as concurrent controls.

Results indicate that, under the conditions tested, the diesel exhaust did not increase the mutation frequency of the

exposed flies (F_2 rate = 0.30%, F_3 rate = 0%) when compared to the concurrent controls (F_2 rate = 0.37%, F_3 rate = 0.15%).

INTRODUCTION

Within the past few years energy conservation has become a major issue throughout the world. While new sources of energy are being sought and developed, old sources are being modified for more efficient and greater economy. As a result, new emphasis may be placed on the diesel engine and its role in mass transportation and various industrial applications. Therefore, because of the large population potentially exposed to the emissions of such engines, knowledge of any associated hazards is vital. Of particular importance is knowledge of any mutagenic effects of diesel emissions. The purpose of the study described in this paper was therefore to use a standard bioassay to evaluate the potential mutagenic effects of diesel exhaust. Specifically, the Drosophila sex-linked recessive lethal bioassay (1), an excellent screen for genetic hazards, was used in this investigation. This bioassay is a useful system for detecting most types of genic damage and has been used to study mutagenic effects of a number of gases and aerosols.

At the request of the United States Environmental Protection Agency, this Drosophila bioassay was performed at the E.P.A. facility (Center Hill Laboratories, Cincinnati, Ohio) utilizing the available resources of ongoing diesel exhaust inhalation experiments. This study was an effort to extract additional data from these chronic diesel studies and at the same time,the intent was not to affect the integrity of the studies in progress by altering such variables as exposure time or concentration. Thus the current study was limited to an 8 hour exposure at the concentration being tested.

EXPERIMENTAL PROCEDURE

Exposure Regimen

Approximately 200 two to five day old wild type male fruit flies (Oregon-R) were exposed to an air stream consisting of diesel engine exhaust gases diluted five-fold with filtered ambient air. The diesel engine used in this study was a 6 cylinder Chrysler-Nissan engine (198 cu. in. displacement) with automatic transmission and dynamometer. A complete description of the operational parameters of this engine is found in Hinners et al. (2). Gross particulates were removed from the ambient dilution air by an MSA-CBR Filter* unit containing high efficiency (99.9% >0.3 microns) HEPA filters. The flies were held in a specially fabricated stainless steel cage (Wire Cloth Company, Cambridge, Maryland).

Mating and Scoring

Randomly selected 100 exposed and 100 control flies were individually mated to two 3 to 5 day old virgin Muller-5 (In[1] sc^{S1L} sc^{8R} + S, sc^{S1} sc^{8} w^{a} B) females (3) on the second day post-exposure. All flies, throughout the study, were kept incubated at a constant 25°C within 8-dram (~30 ml) shell vials containing an instant Drosophila medium, Formula 4-24 without dye (Carolina Biological Supply Co., Burlington, NC, 27215). Carbon dioxide was used to anesthetize the flies. Pure CO_2 gas (99.9%) regulated from a cylinder, passed through a cotton-filled box (3 X 5 X 1 inch) having a platform constructed of fine wire mesh. The flies were allowed to deposit their eggs for the next two days. On the fourth day post-exposure, the females were transferred to new vials containing fresh media and allowed to continue egg deposition. Concurrently, the males were individually placed into fresh vials in order to maintain a record of parental lines. These two "sub-broods" represented brood I, i.e., the sperm used in these matings were mature spermatozoa (post-meiotic) at the time of exposure (4).

The males were again individually mated to fresh virgins on the eighth day post-exposure. As before, the females were permitted to deposit their eggs for the next two days before being transferred to fresh vials. These two "sub-broods" represented brood III, i.e., the sperm used in these matings were in the spermatocyte (pre-meiotic) stage of spermatogenesis at the time of exposure (4).

The parents (P_1) of both second "sub-broods" were removed before emergence of the F_1 generation. Ten F_1 females were selected at random from each P_1 vial (from the original matings) up to 70 vials/brood and individually mated to two of her Muller-5 brothers. Therefore, a total of 700 F_1 matings/Brood/control and exposed groups were performed. The resultant F_2 cultures were scored for a sex-linked recessive lethal event according to standard procedures (1). An F_3 test was performed by selecting one female from every other F_2 culture vial and mating her with two of her Muller-5 brothers. The resultant F_3 cultures were scored in the same manner as the F_2 cultures.

RESULTS

Comparative analyses of the atmospheres of the Drosophila chamber and the 3 cubic meter chamber (Table 1) revealed that the concentration values for most atmospheric components were lower in the Drosophila chamber with the exception of the hydrocarbon fraction. Specifically, the Drosophila chamber showed 54.8% of the CO_2 level of the 3 cubic meter chamber, 55.2% of the CO level, 124.7% of the HC level, 29.5% of the NO level, 76.3% of the NO_2 level, 43.5% of the SO_2 level and 30.1% of the particulate level of the three cubic meter chamber.

The fly cage (Figure 1) was cylindrical in shape (8½ X 3½ inches) with one end terminating into a cone with a quick connect/disconnect port allowing rapid ingress and egress to and from the cage interior; the opposite end, together with the remainder of the structure, was enclosed with 24 by 24 mesh, 0.04-inch wire diameter, stainless steel screen. The cage was placed within a glass chamber (2000 ml Reaction Flask, Ace Glass Inc.). The fly cage was secured within the chamber by a preformed band of silicone rubber which made an occlusive seal between the glass wall and the screen, thus allowing for all the diesel exhaust mixture to pass through the cage. This air was pulled by vacuum pump through this system at a rate of 1.06 liters per minute. The source of the engine exhaust mixture was a 3 cubic meter stainless steel exposure chamber (Figure 2) that was being used for repeated exposures of laboratory animals to diesel exhaust. These animals were present in the chamber during the 8 hour exposure period. The diesel engine was alternately turned on and off at 15-minute intervals throughout the 8 hour exposure period. An identical exposure system was used for an equal number of male flies. However, these flies served as concurrent negative controls exposed only to the filtered ambient air. Both the control and the exposed flies were randomly collected from the same stock of 2 to 5 day old males.

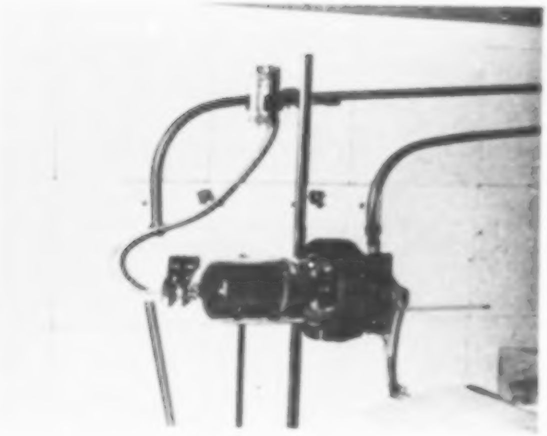

Figure 1
Fly Chamber with Cage

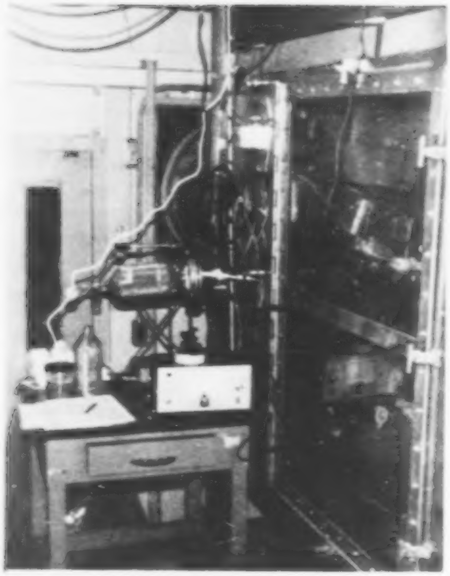

Figure 2
Fly Chamber Connected to 3 Cubic Meter Chamber

Sampling and Analysis

Characterization of the atmosphere within the 3 cubic meter chamber was compared to the atmosphere sampled after passing through the Drosophila chamber (Table 1). Analyses were performed by E.P.A. personnel of the Center Hill Laboratories, with the exception of the gravimetric particulate sample. An integrated air sample of the fly chamber, collected in a preconditioned mylar plastic sampling bag, was taken over the entire exposure period. The CO and CO_2 levels were measured by a Beckman Infrared Analyzer, hydrocarbons by hydrogen flame ionization on a Beckman Total Hydrocarbon Analyzer, SO_2 by pulsed fluorescence on a Thermo-electron SO_2 Analyzer, and NO/NO_2 by a chemiluminescent (ozone reaction) method using a Thermo-electron NO/NO_2 Analyzer. Particulate concentrations were determined gravimetrically using 37 mm glass fiber filters mounted between the fly chamber and the sampling bag. Temperature and percent relative humidity were recorded hourly. Temperature was measured with a thermometer mounted within the Drosophila chamber. Relative humidity of the Drosophila chamber was measured using a Weather Measure Corp. Relative Humidity Indicator, Model HMI-14. Temperature and humidity readings were not available from the 3 cubic meter chamber.

Scoring of the F_2 cultures resulted in three lethals found in 678 vials scored from brood I in the control group compared with three lethals in 670 vials of the exposed group (see Table 2). Controls from brood III yielded two lethals in 676 vials compared to one lethal in 680 vials of the brood III exposed group. No lethals were found in the F_3 cultures of brood I of both the control (334 vials) and the exposed (also 334 vials) groups; only one lethal was found in the brood III control group (346 vials) while no lethals were found in the exposed group (336 vials).

The combined F_2 (broods I and III) results of five lethals/1354 vials scored for the control group gives a "background" mutation rate of 0.37%. In comparison, the combined F_2 results in the exposed group of four lethals/1350 vials yield a rate of 0.30%. The combined F_3 (broods I and III) results of one lethal/680 vials gives a rate of 0.15% for the control group while the exposed group had no lethals recorded in 670 vials scored.

When the F_2 and F_3 totals are combined, the control group shows a rate of 0.29%, six lethals/2034 vials, versus a rate of 0.20%, four lethals/2020 vials, for the exposed group.

TABLE 1

CHARACTERIZATION OF CHAMBER ATMOSPHERES

Component Measured	3 cu m Chamber	Drosophila Chamber
CO_2	0.31%	0.17%
CO	22.1 ppm	12.2 ppm
HC	9.3 ppm	11.6 ppm
NO	12.9 ppm	3.8 ppm
NO_2	3.8 ppm	2.9 ppm
SO_2	2.3 ppm	1.0 ppm
Particulate	7.3 mg/m^3	2.2 mg/m^3
Relative Humidity	-	29.1% + 1.5
Temperature	-	22.3°C + 0.2

TABLE 2

RESULTS

SEX-LINKED RECESSIVE LETHAL TEST

F_2 Brood	Control No. lethals/No. scored	Exposed No. lethals/No. scored
I Sub-brood A	0/331	2/332
Sub-brood B	3/347	1/338
III Sub-brood A	1/342	0/346
Sub-brood B	1/334	1/334
Total F_2	5/1354 = 0.37%	4/1350 = 0.30%

F_3 Brood	Control No. lethals/No. scored	Exposed No. lethals/No. scored
I Sub-brood A	0/170	0/171
Sub-brood B	0/164	0/163
III Sub-brood A	1/174	0/167
Sub-brood B	0/172	0/169
Total F_3	1/680 = 0.15%	0/670 = 0%
Total F_2 + F_3	6/2034 = 0.29%	4/2020 = 0.20%

DISCUSSION

The number of recessive lethals found in the F_2 and the F_3 treatment groups of both broods was smaller than in the control groups, indicating a lack of mutagenic activity exhibited by the diesel exhaust under the conditions of this study.

The effluent concentrations of some of the diesel exhaust components of the fly chamber differed substantially from those measured in the 3 cubic meter chamber. Much of the particulate loss may have been a result of adsorption along the network of fine wire mesh of the cage or to the walls of the glass chamber. A set of particulate samples was taken concurrently at a later date to examine this possibility, however, no significant difference in concentrations was found between the sample taken directly from the inhalation chamber and the sample taken immediately after the fly chamber and cage. Other diesel exhaust components may have been adsorbed onto the walls of the chamber or scavenged by the rubber tubing connections before reaching the sampling devices.

Two factors must be considered for the proper interpretation of the results of this study. First, the number of flies used in this test is capable of detecting moderate or stronger mutagens exhibiting 3 to 5 or more times the background or control mutation rate (albeit a low rate of approximately 0.1% to 0.6%-many authors); a weaker activity of twice the background rate or less could have eluded detection. However, there is no evidence in the data that suggests that weak activity might have been present, i.e., more mutations were found in the control groups than in the treated groups. A second, and perhaps more relevant, fact is that the objective of the recessive lethal test is more to reveal qualitative information rather than quantitative or dose-response type information (5). Therefore, a higher concentration should be administered to the flies for a more thorough assessment of the mutagenic potential of the diesel exhaust. Specifically, the concentration level used should result in a significant degree of mortality or sterility in the flies after exposure or, if this is mechanically unachievable, the exposure concentration should represent the maximum level that can be consistently generated and maintained over the duration of the desired exposure period. The duration of exposure can be extended up to approximately 24 hours and still allow for brood analysis with minimal overlapping of the separate stages of spermatogenesis. In addition, more brood types could be investigated to aid in the detection of any stage specificity effect that might otherwise escape detection.

Furthermore, it has been demonstrated that some of the components of diesel exhaust have been shown to be mutagenic either alone or synergistically in the combined treatment with other substances. Carbon monoxide alone (95% CO, 5% O_2/6 hours) did not increase sex-linked recessive lethals in Drosophila, but when combined with azide or potassium cyanide, a significant increase in recessive lethals was found (6). Azide and potassium cyanide, either alone or in combination, did not show such an increase. Three to four percent nitric oxide (NO) delayed spermatogenesis, increased dominant lethality in Drosophila, and increased the percentage of sex-linked recessive lethals in γ-irradiated Drosophila (7). Sulfur dioxide (SO_2) alone exhibited a significant increase in the mutation rate above spontaneous levels in Tradescantia (8). These facts, coupled with the fact that the hydrocarbon fraction of the exhaust may contain potential carcinogens and/or mutagens, reinforce the need for an additional, more challenging, dose for administration to Drosophila.

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* Mention of a company or product name does not constitute endorsement by NIOSH.

General Discussion

S. SODERHOLM: It is rather off the main point of your talk, but there is a puzzling difference in concentration especially gas concentration between the inhalation chamber and the Drasophila chamber. The fact that it didn't occur a second time suggests that you had a leak in the line somewhere and a small negative pressure in the main chamber. This can occur fairly simply.

R. SCHULER: Yes, one of our disappointments was the inability to pinpoint the exact reasons why we had differences.

R. SCHRECK: Could you tell us the route of entry of the particle into the fly? Do you feel it comes in through their tiny respiratory passageways in the side, and what do you expect the dose is per animal?

R. SCHULER: I don't know the exact dose, however, the route of entry is through the spiracles on the side of the abdomen of the fly. They can selectively open and close these spiracles. However, they only do this when they are challenged with a very irritating type of exposure. Normally, this is demonstrated with erythromycin. However, the fact that they are readily put under anesthesia by carbon dioxide in a fraction of a second indicates that the entry is rapid.

D. BRUSICK: It might be worth noting that the Drosophila can also ingest the particles. It has been well documented mutations can be induced in Drosophila by ingestion. Do you feel that relates in any way to what you observed?

R. SCHULER: Again as I emphasized, I would prefer to be doing a much higher and more challenging dose in terms of concentration and exposure time before I make any definite statements as to why we got these results. We were limited by the conditions of an ongoing experiment.

METAPHASE ANALYSIS, MICRONUCLEI ASSAY AND URINARY MUTAGENICITY ASSAY OF MICE EXPOSED TO DIESEL EMISSIONS

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ABSTRACT

Female Swiss mice were exposed 8 hours per day to diesel exhaust for 1, 3 and 7 weeks. Urine was collected overnight for 4 days prior to sacrifice while the mice continued to be exposed for eight hours during the day. The presence of mutagens was determined by the Ames *Salmonella* test. One hour prior to sacrifice each mouse received 1 mg/kg colcemide. After sacrifice, the marrow from each femur was obtained. The marrow from one femur was used to prepare slides for metaphase analysis and the other for micronuclei assay. Other mice received i.p. 50 mg/kg cyclophosphamide 24 hours prior to sacrifice or 1 umole/kg benz(a)pyrene in each of four daily doses prior to sacrifice and served as positive controls. The Ames *Salmonella* assay of the unconcentrated urine after 1, 3 and 7 weeks and concentrated urine after 7 weeks exposure to diesel exhaust did not significantly vary from clean air controls. Results In the micronucleus test, and metaphase analysis cyclophosphamide produced a strong positive response and the 7 week diesel exposure was not different from clean air controls.

The genotoxic hazard of exposure to diesel exhaust was investigated in mice. Diesel exhaust contains thousands of organic chemicals some of which are known carcinogens and mutagens including polycyclic aromatic hydrocarbons. The high particle concentration of diesel exhaust results in the sequestering on the particles of much of the polycyclic aromatic hydrocarbons. In order for the genotoxic agents in diesel exhaust to exert their activity they must be deposited in the lungs, eluted from the particles and distributed throughout the body.

Genotoxic agents can produce two types of deleterious alterations, i.e. mutagenic and clastogenic effects. Agents that cause mutations can be detected in the urine of animals by the Ames *Salmonella* assay (Legator, et al. 1977). Clastrogens can be detected by metaphase analysis for breaks in chromatids and chromosomes (Cohen and Hirschhorn, 1971) and by the micronuclei assay (Schmid, 1977). Micronuclei result from the membrane encapsulation of chromosomal material that did not segregate with the spindle apparatus as the result of chromosome breakage or disruption of the spindle apparatus. In this communication, we report investigations that attempted to detect the absorption and systemic distribution of genotoxic agents in mice exposed to diesel exhaust by a combined protocol consisting of 1) metaphase analysis, 2) micronuclei and 3) urinary mutagenicity.

MATERIALS AND METHODS

Ten female Swiss mice (Charles River) were assigned to the various treatment groups. Those exposed to diesel emissions were exposed 8 hours per day, 5 days per week, at the U.S. EPA Health Effects Research Laboratory, Cincinnati, Ohio. The diesel emission was produced by a Nissan 6 cylinder run on the Federal Short Cycle. The exhaust exposure contained 6-7 mg/m^3 particles at a 1:18 dilution (Hinners, 1980).

Control groups were exposed under similar conditions without the diesel emission. Urine samples on the 4 days prior to sacrifice were collected overnight in a brown bottle embedded in salt-ice and kept at (-) 15 to (-) 10°C. On the day of sacrifice, colcemide (1 mg/kg) was injected i.p. 30 minutes before the animals were killed by cervical dislocation. The marrow from one femur was used for the preparation of slides for micronuclei and the other for slides for metaphase analysis according to Connor et al. (1979). The urine samples were either (a) tested directly with 5 strains of *Salmonella typhimurium* using method described by Connor et al (1979) or (b) concentrated on an XAD-2 column according to the procedure of Yamasaki and Ames (1977). The samples were concentrated 20-fold and 50 ul of concentrate were assayed with and without B-glucuronidase (100 units per plate) using *S. typhimurium* TA98 and TA100. The unconcentrated urine samples were tested at 200 ul per plate in the presence of

B-glucuronidase. Additional groups of control mice received i.p. 1 cyclophosphamide at 50 mg/kg, one time; 2) benz(a)-pyrene at 1 umole/kg four times; 3) water (vehicle for cyclophosphamide), one time; and 4) corn oil (vehicle for benz(a)-pyrene), four times. The slides and the frozen urine samples were transported to the University of Texas Medical Branch for analysis. Figure 1 outlines the treatment and analysis protocol described in this paper.

RESULTS

Metaphase analysis was performed on only the 7-week diesel exhaust exposures and controls. There was no indication of chromosomal or chromatid damage (Table 1). Cyclophosphamide controls produced many damaged cells and benz(a)pyrene at the low dose used was inactive. The low dose of benz(a)-pyrene was chosen as an approximation of the dose of benz(a)-pyrene expected to result from the diesel exhaust exposure.

The micronuclei bioassay was performed on mice exposed for 1, 3 and 7 weeks to diesel exhaust. There was no significant difference in the number of micronuclei in polychromatic erythrocyctes from controls and mice exposed to diesel exhaust (Table 2). Cyclophosphamide did induce micronuclei while benz(a)pyrene was inactive. With both the unconcentrated and the concentrated urine samples, all the clean air and diesel exhaust exposure values were within the expected ranges for the various strains (Tables 3 and 4). Where possible, urine was collected sequentially at 1, 3 and 7 weeks from the same mice exposed to either clean air or diesel exhaust. Again, the cyclophosphamide and not the benz(a)pyrene was detected in both the unconcentrated and the concentrated samples with strains TA1535 and TA100.

DISCUSSION

We attempted to determine the absorption and systemic distribution of the genotoxic agents, mutagens and clastogens, presence in diesel exhaust emissions. Mice were exposed to diesel exhaust and evidence of systemic distribution examined by the combined protocol of 1) metaphase analysis, 2) micronuclei assay and 3) urinary mutagenesis. The metaphase analysis and the micronuclei assay detect chemicals capable of breaking chromosomes and chromatids and disrupting the spindle apparatus. The urinary mutagenicity assay detects the presence of chemical mutagens. These assays have the advantage of being in vivo systems that allow for the metabolic and pharmacokinetic factors which control the effectiveness of the mutagens present in diesel exhaust.

The combined protocol used was unable to detect the systemic distribution of genotoxic agents resulting from diesel

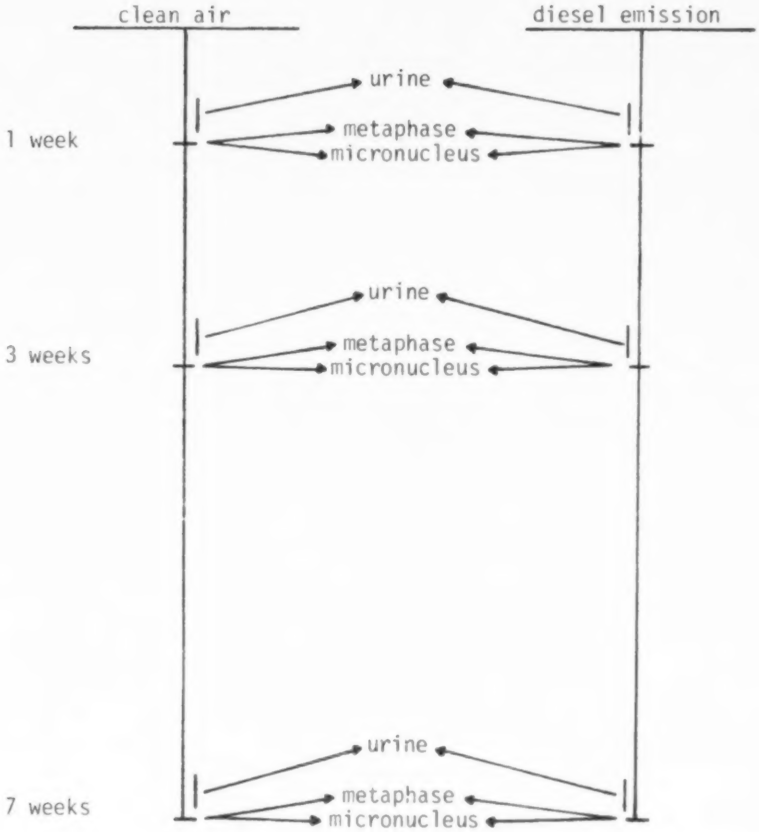

Figure 1. Treatment and Testing Protocol.

TABLE 1

METAPHASE ANALYSIS IN MICE EXPOSED TO DIESEL EXHAUST

Exposure	Time	No. Metaphases Analyzed	Chromatid Breaks	Chromosome Breaks	Concentric Figures	Fragments
Control	7 weeks[a]	750	3	0	2	1
Diesel	7 weeks	950	1	0	2[d]	0
[b]Cyclophosphamide		250	200 (cells grossly damaged)			
[c]Benzo(a)pyrene		750	7	2	4	3

[a] mice were exposed 8 hours per day, 5 days per week, 10 mice per group.

[b] 50 mg/kg, i.p., 4 times

[c] 1 μmole/kg, i. p., 4 times

[d] Robinsonian Translocations

TABLE 2

Micronucleus Test in Mice Exposed to Diesel Exhaust

Exposure	Time	Micronuclei per 1000 Polychromatic Erythrocytes[d] Mean	S.D.
Diesel	1 week[a]	1.0	0.82
Control	1 week	0.4	0.70
Diesel	3 weeks	0.9	1.20
Control	3 weeks	1.1	0.74
Diesel	7 weeks	0.9	0.99
Control	7 weeks	1.7	1.34
Cyclophosphamide[b]		31.6	18.79
Benzo(a)Pyrene[c]		0.8	0.79

[a] mice were exposed 8 hrs per day, 5 days a week for numbers of weeks specified, 10 mice per group.

[b] 50 mg/kg, i.p., 1 time

[c] 1 μmole/kg, i.p., 4 times

[d] 1000 cells counted per animal

TABLE 3

Mutagenicity of Urine Samples of Mice Exposed to Diesel Exhaust and Assayed with Salmonella typhimurium

		Histidine revertants per plate[a]				
Exposure	Time	TA1535	TA1537	TA1538	TA100	TA98
Clean air	1 week (4)[b]	5[c]	2	2	44	6
	3 weeks (6)	9	1	2	43	9
	7 weeks (3)	2	2	10	22	15
Diesel	1 week (2)	4	0	0	44	11
	3 weeks (6)	10	1	8	45	8
	7 weeks (3)	6	4	9	23	13
Cyclophosphamide	(1)	370	3	9	345	16
Control (water)	(1)	7	0	14	27	5
B(a)P	(1)	0	1	6	10	17
Control (corn oil)	(1)	3	0	10	26	0

[a] Control values for each strain have been substracted. 200 μl of urine added per plate. 100 units of β-glucuronidase added per plate. 2 plates per sample

[b] Number of samples tested

[c] Mean of samples

TABLE 4

MUTAGENICITY OF CONCENTRATED URINE SAMPLES OF MICE EXPOSED TO DIESEL EXHAUST AND ASSAYED WITH SALMONELLA TYPHIMURIUM

Exposure	Time	Histidine revertants per plate[a]		
		B-glu	TA100	TA98
Clean air	1 week	-	10	15
		+	19	10
	3 weeks	-	9	13
		+	11	9
	7 weeks	-	44	21
		+	24	24
Diesel	1 week	-	19	14
		+	25	11
	3 weeks	-	0	16
		+	26	11
	7 weeks(3)[c]	-	33	10
	(3)	+	27	19
Cyclophosphamide		-	308	4
		+	303	11
B(a)P		-	10	0
		+	0	0

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[a] Control values for each strain have been subtracted, 100 units B-glucuronidase added, as indicated.

[b] Urine was concentrated 20-fold by XAD-2 column and 50 ul of concentrated urine added per plate.

[c] Number of samples tested is one if not given, in which case the values are the average of the samples tested.

exhaust emissions. The failure to detect genotoxic agents could have resulted from (a) the level of genotoxic agents in diesel exhaust was too low to be detected or (b) the route of exposure (inhalation) and (c) the absorption of the genotoxic agents onto particles did not allow them to be absorbed, distributed and metabolized. The combined protocol is sensitive to many genotoxic agents including cyclophosphamide (Connor, et al., 1979; Connor et al., 1980; Legator et al. unpublished results). However, the combined protocol was not sensitive to benz(a)pyrene at 1 umole/kg for 4 days. This dose of benz(a)pyrene was chosen to approximate the dose of benz(a)pyrene resulting from the diesel exhaust exposure. Benson et al, 1978 have reported that 100 mg/kg (400 umole/kg) i.m. of benz(a)pyrene could be detected in the urine. Siou et al, 1977 have reported that 100-500 mg/kg (400-2000 umole/kg of benz(a)pyrene induced micronuclei and Balser and Rohrborn, 1976 reported the induction in Chinese hamsters of chromosomal aberrations.

It would appear that the bioassays employed in the combined protocol are not sensitive enough to benz(a)pyrene to detect the amount of benz(a)pyrene present in diesel exhaust. However, it can not be ruled out that genotoxic agents are present in diesel exhaust in sufficient concentration to be detected by the employed bioassays if they are not sequestered on particles. We plan to further investigate this possibility by testing under the combined protocol diesel exhaust particles and their extracts.

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General Discussion

D. ROUNDS: Can you tell me how the benzo(a)pyrene was administered in the SCE Study?

M. PEREIRA: It was done by IP injection.

D. ROUNDS: Have you tried doing an IP injection of the diesel particles?

M. PEREIRA: Not yet; we are planning to do that.

A. BROOKS: I am a little curious as to how you are going to increase the concentration very much higher than what you already have?

M. PEREIRA: The dilution ratio of diesel exhaust in the studies we have reported was one to 18. This concentration has since been increased at the EPA Center Hill Facility.

W. PEPELKO: Yes, a dilution ratio of about one to 18 was used. We are presently using a dilution ratio of one to nine which doubles our particulate concentration to 12 millograms per cubic meter.

A. BROOKS: Have you tried any other cell systems where chromosome aberrations persist chromosomic, since both the systems that you are using are rapidly proliferating and the amount of chromosome damage you see would be dependent on this rate? In other words, at each cell division, the abnormal chromosomes are eliminated so that you are never going to achieve a very high total chromosome aberration frequency in that system.

M. PEREIRA: That is why we believe more in the hyperkinetic system.

IN-VIVO DETECTION OF MUTAGENIC EFFECTS OF DIESEL EXHAUST BY SHORT-TERM MAMMALIAN BIOASSAYS

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ABSTRACT

Male chinese hamsters were exposed to diesel exhaust and clean air for six months at the Center Hill Facility of the U.S. Environmental Protection Agency in Cincinnati, Ohio. The animals were kept in specially constructed inhalation chambers and exposed to clean air or diesel exhaust for eight hours daily. The animals were sacrificed and slides prepared to study the mutagenic effects of diesel exhaust by four in vivo short term mammalian bioassays. Sperm morphology bioassay revealed a 2.67 fold increase in sperm abnormalities in the animals exposed to diesel exhaust as compared to those exposed to fresh air. Micronucleus bioassay revealed a 50% increase in the number of micronuclei in polychromatic erythrocytes obtained from animals exposed to diesel exhaust. However, no increase in sister chromatid exchange or chromosomal abnormalities was observed in bone marrow cells of animals treated with diesel exhaust. During these studies a decrease in mitotic index was observed in animals treated with diesel exhaust.

Introduction

The projected increase in the number of diesel cars to be manufactured make it imperative to study the mutagenic effects of diesel exhaust. Diesel exhaust is an extremely complex mixture of inorganic and organic compounds. Some of the organic compounds in diesel exhaust are known mutagens and carcinogens in the free form. However, in diesel exhaust the chemicals are sequestered on particulate matter. The genotoxic effects of these particle-bound chemicals are unknown.

The mutagenic potential of these chemicals in diesel exhaust is influenced by pharmacokinetic and metabolic factors that can only be addressed by _in vivo_ studies. Synergistic effects on biotransformation pathways, pharmacokinetics and interactions at the target organs may be important in determining the overall effects on the animals. _In vivo_ studies of the genotoxicity of diesel exhaust reported in this communication were designed to permit full interplay of these factors.

It has been shown that there is an increase in the frequency of abnormal sperm in mice exposed to various carcinogenic and mutagenic chemicals (1,2). Bruce _et al._ (3) reported increase in the frequency of abnormal sperms when animals were exposed to X-ray at a very low dose of 30 rads. Since carcinogens and mutagens cause genetic damage, it is likely that the observed sperm abnormalities are a result of genotoxic effects (4).

It has been observed that there is an increase in the frequency of micronucleated polychromatic erythrocytes in animals exposed to various carcinogenic and mutagenic chemicals (5, 6). Wild (7) also reported that the induction of micronuclei for many common chemical mutagens and carcinogens correlated with results from other mutagenicity and carcinogenicity tests. The micro-nucleus test is a procedure to detect chromosome fragments that did not segregate with the spindle apparatus. The fragments are not incorporated into the cell nuclei but rather form smaller nuclei called micronuclei. Micronuclei are found in a variety of cells; however, quantitation of micronuclei is most easily carried out in erythrocytes since the micronuclei remain in the red blood cells even after the expulsion of nucleus by the erythroblasts. Consequently, the micronuclei are easily detected.

Various known carcinogens and mutagens have been reported to increase significantly the frequency of sister chromatid exchange (SCE) both in vitro (8) and in vivo. The use of 5-bromodeoxyuridine (BUdr) and 33258 Hoechst staining techniques for the detection of sister chromatid exchange induction in cultured cells has proven useful for highly accurate analysis (9). The relevance and flexibility of SCE analysis was greatly enchanced, however, when the _in vivo_ induction of SCE in chick embryos (10), mouse spermatogonia (11), and mouse bone marrow (12) was described. Since in vivo induction of SCE is an extremely sensitive bioassay (13), we have studied the mutagenic effects of diesel exhaust on male Chinese hamsters using this test.

The induction of chromosomal aberrations has been used as an indicator for mutagenic agents in the environment (14). This bioassay shows excellent correlation when used with known mutagens and carcinogens (15). It has been clearly shown by many investigators (16) that chemicals which induce mutations at specific loci in eukaryotic cells invariably result in cytologically observable chromosomal damage expressed as aberrations. In the present investigations we have determined the effect of diesel exhaust in Chinese hamster on 1) sperm morphology; 2) the induction of micronuclei in bone marrow cells; 3) the induction of sister chromatid exchange in bone marrow cedlls; and 4) the cytology of bone marrow cells.

Materials and Methods

Male Chinese hamsters were exposed to diesel exhaust daily (8 hrs) for 6 months at the Center Hill Facility of U.S. Environmental Protection Agency at Cincinnati, Ohio. Control animals were exposed to fresh air. The procedurs for various bioassays are described below:

1. Sperm Morphology Bioassay

The animals were sacrified by cervical dislocation and the cauda epididymes removed. A sperm suspension was prepared by mincing the cauda in 2 ml of phosphate buffered physiological saline. It was then pipetted a few times to insure uniform distribution of the sperm cells in the suspension. A fraction of each suspension was mixed (10:1) with 1% Eosin Y in alcohol. After 30 minutes, a few drops of this suspension were used to make a smear. Five slides were prepared for each animal. The slides were allowed to air dry and mounted with a coverslip using Permount. For each animal 500 sperm cells were observed at 400-fold magnification with blue-green filter and the percentage of abnormal sperm/animal scored from coded slides.

2. Micronucleus Bioassay

Ten animals exposed to diesel exhaust and ten animals exposed to fresh air were used for the experiment. After sacrifice of the animals by cervical dislocation, the femur was removed by cutting through tibia and pelvis; the bone was freed from muscle. Then, the epiphyseal part of the bone was torn from the rest of tibia. The proximal end of the femur was then cut off so that a small opening to the bone marrow canal could be seen.

Centrifuge tubes were filled with fetal bovine serum up to the rim. A 1 cc syringe with mounted needle was used to aspirate about 0.2 ml of serum. Then, the needle was inserted a few millimeters deep into the proximal part of bone marrow. With the femur completely submerged, the syringe was gently aspirated and flushed several times. The process was repeated for the distal part of the bone marrow.

The centrifuge tubes were spun at 1000 rpm for 5 minutes. Supernatant was removed from the tubes using a Pasteur pipette and the serum was completely removed. The sediment was gently aspirated into the capillary portion of a siliconized Pasteur pipette, and a small drop was transferred to a slide and spread out using a cover slip held at an angle of 45 degrees. These preparations were air dried for 24 hours.

The preparations were stained for 3 minutes in undiluted May-Gruenwald solution, and 2 minutes in May-Gruenwald diluted with double distilled water 1:1, and rinsed in distilled water and stained again for 10 minutes in Giemsa, diluted with water 1:6. Then, the slides were rinsed under tap water and blot dried with filter paper. The dried slides are then cleared in Xylene for 5 minutes and mounted in Permount.

The slides were coded and screened to determine the number of micronuclei per 1000 polychromatic erythrocytes per animal.

3. In vivo SCE Bioassay

Six animals were exposed to diesel exhaust, six animals to clean air and four animals to benz(a)pyrene (BaP) (Sigma) as a positive control. A pellet of 70-75 mg 5-bromodeoxyuridine (Sigma) was implanted subcutaneously in the abdominal area (17). Care was taken not to disturb the peritoneal membrane. Ether was used to anesthetize the animals and #4 Ethicon nylon sutures were used to close the incision made for implanting the pellet. Animals in the experimental group were given thirty minutes to recover from the ether. The animals were then returned to diesel exhaust chamber. Animals in the positive control group were injected intraperitoneally with B(a)P at 100 mg/kg body weight two hours following implantation. Animals in the negative control group were given thirty minutes to recover from the ether and then returned to their clean air chambers.

Twenty-four hours following implantation, each animal was injected with colchicine (Sigma) at 10 mg/kg body weight. Two hours following treatment with colchicine, each animal was sacrificed by cervical dislocation. After sacrifice, the femur was removed from each leg by cutting through the tibia and pelvis. Using tissue paper, all muscle was removed from the bone. Then the epiphyseal portions of the femur were clipped leaving the diaphysis with the bone marrow canal exposed at both ends. The bone marrow cells were flushed from the canal using physiological saline solution warmed to 37°C. This suspension was centrifuged for 10 minutes at 1000 rpm. The saline was then discarded, and the cells were resuspended in 0.075M KCl for 30 minutes at 37°C. The cells were fixed in methanol-glacial acetic acid (3:1) and were allowed to stand in the first fix for 20 minutes and in the second fix overnight. After one change of the fixative, on the following day, the cells were placed on cold slides and were allowed to air dry.

After air drying for 24 hours, differential staining was performed by (8) allowing the slides to stand in the absence of light for 15-18 minutes in a solution of 33258 Hoechst (5mg/100 ml H_2O), American Hoechst Corporation, (2) dipping in distilled water and blotting dry (3) applying six drops of McIlvaines buffer solution (ph=8.0), coverslipping, and placing on a slide tray warmed to 50°C with a 20 watt black light mounted 5 cm above the slides, and exposing the slides to the black light for 22 minutes, (4) floating the coverslip off by dipping in distilled water (5) blotting the slides dry and allowing them to stand for 2 hours, and (6) placing the slides in 2% Giemsa in Gurr buffer solution (pH=6.8) for 10 minutes. Slides were coded for observations.

Twenty-five metaphase cells from each animal were evaluated for SCE. The mitotic index was determined for each animal by counting the number of metaphase cells observed per 1000 cells. Five thousand cells were counted from each animal.

4. Cytological Aberration Bioassay

For these studies, the slides prepared for SCE were used. Twenty-five metaphase cells from each control and diesel exhaust treated animal were evaluated for polyploidy, various chromosomal and chromatid aberrations.

Results and Discussion

1. Sperm Morphology Bioassay

Various abnormalities in sperm shape were observed both in control and diesel exhaust treated animals (Fig. 1) The

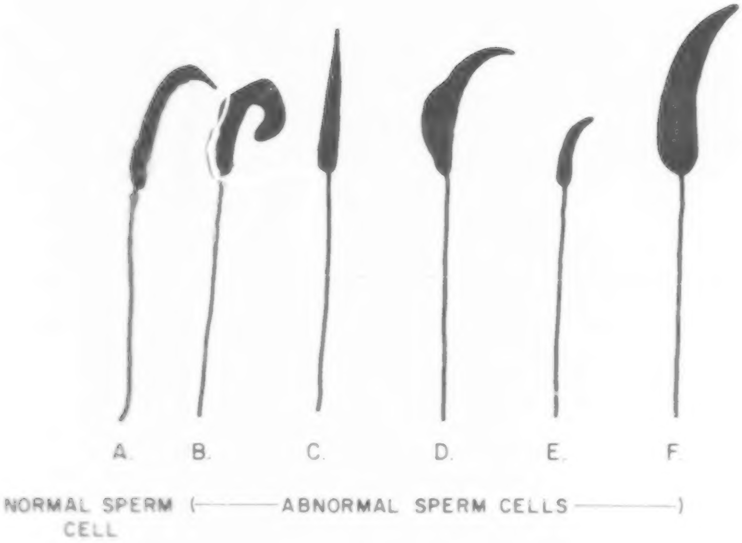

FIGURE 1. Normal and abnormal shape of Chinese hamster sperm cells.

observed abnormalities included curled head, amorphous head, non-sickled shaped head and abnormally small size of head usually accompanied by curled head. Table I shows the results for each animal and the pooled data. The percentage of abnormal sperm significantly increased in animals exposed to diesel emissions. Both the diesel exhaust exposed animals and controls had a very low frequency of abnormal sperm. The frequency of sperm abnormalities, while varying among different inbred strains, is relatively constant within any particular animal strain (18). Thus, the differences in frequency of abnormal sperm can be significant even in such small percentages as observed in this study; however due to the small sample size these results should be considered preliminary until reported. In a larger sample of mice exposed to diesel exhaust for 7 weeks, no increase in the frequency of abnormal sperm was observed (19).

2. Micronucleus Bioassay

There was some increase in the frequency of micronuclei in animals exposed to diesel exhaust for 6 months. The treated animals showed 0.3% to 1.3% cells with micronuclei as compared to controls which had 0.2% to 0.6% cells with micronuclei (Table II). The mean for the untreated animals was 0.4% while it was 0.69% for the animals exposed to diesel. According to the Student t test procedure the data showed significant increase in micronuclei in hamsters exposed to dieset exhaust at the P=0.2 level. In mice, exposure to diesel exhaust for only 7 weeks did not result in an increase in micronuclei (20). Whether the lack of an effect of diesel exhaust exposure in mice on the induction of micronuclei is a species variation or a result of the shorter duration of exposure is under further investigation.

3. *In vivo* SCE Bioassay

The mitotic index of animals exposed to diesel exhaust was clearly decreased, but no effect was observed on the frequency of SCE (Table III and IV). The evaluation of metaphase cells from animals in this group was hampered by the clumping of metaphase chromosomes. It has been noted that these animals weighed more than the other animals used in this evaluation. We have previously observed a similar clumping effect in obese animals. Further investigation will be required to determine if both the decreased mitotic index and the problem of clumped metaphases was due to the weight of the animals or the prolonged exposure to diesel emissions. The possibility of a "combined effect" must also be entertained.

TABLE I. INDUCTION OF SPERM ABNORMALITIES IN CHINESE HAMSTERS EXPOSED TO DIESEL EXHAUST

TREATMENT	DURATION OF TREATMENT	ANIMAL NUMBER	# OF ABNORMAL SPERM (%)
CLEAN AIR (CONTROL)	6 MONTHS	1	0.4
		2	0.4
		3	0.2
		4	0.2
		5	0.2
		6	0.4
		7	0.6
		8	0.6
		9	0.2
		10	0.4
			Average = 0.36 ± .05[1]
DIESEL EXHAUST	6 MONTHS	1	1.8
		2	1.2
		3	1.0
		4	1.4
		5	1.2
		6	0.6
		7	0.0
		8	1.0
		9	0.4
		10	1.0
			Average = 0.96 ± .16[2]

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[1] Average ± Standard Error of the Mean.

[2] The Increase was significant with P = 0.0024 by the Student t Test.

TABLE II. INDUCTION OF MICRONUCLEI BY DIESEL EXHAUST IN CHINESE HAMSTER BONE MARROW CELLS

TREATMENT	DURATION OF TREATMENT	ANIMAL #	# POLYCHROMATIC ERYTHROCYTES OBSERVED	% MICRO-NUCLEI
CLEAN AIR (CONTROL)	6 MONTHS	1	1000	0.5
		2	1000	0.3
		3	1000	0.6
		4	1000	0.3
		5	1000	0.2
		6	1000	0.5
		7	1000	0.5
		8	1000	0.4
		9	1000	0.4
		10	1000	0.3
			Average = 0.40 ± .04[1]	
DIESEL EXHAUST	6 MONTHS	1	1000	0.5
		2	1000	0.3
		3	1000	0.9
		4	1000	0.5
		5	1000	1.3
		6	1000	0.7
		7	1000	0.6
		8	1000	0.3
		9	1000	0.9
		10	1000	0.9
			Average = 0.69 ± .10[2]	

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[1] Average ± Standard Error of the Mean.

[2] The Increase was significant with $P = 0.02$ by the Student t Test.

TABLE III. EVALUATION OF THE SCE/CELL AND THE MITOTIC INDEX

TREATMENT	ANIMAL #	WEIGHT (GM)	MITOTIC INDEX	CELLS OBSERVED	SCE/CELL
CLEAN AIR	1	30.2	44[1]	25	5.12 ± 0.5[2]
	2	31.0	39	25	4.36 ± 0.4
	3	34.0	39	25	4.84 ± 0.4
	4	30.1	39	25	5.80 ± 0.4
	5	31.7	41	25	4.72 ± 0.3
	6	29.0	29	25	4.28 ± 0.4
B(a)P	1	32.2	25	25	10.04 ± 1.2
	2	35.4	32	25	9.80 ± 0.7
	3	31.0	27	25	10.04 ± 0.8
	4	29.1	18	25	12.40 ± 1.1
DIESEL EXHAUST	1	39.7	30	25	4.40 ± 0.5
	2	35.2	15	25	4.24 ± 0.5
	3	35.8	33	25	4.68 ± 0.4
	4	38.8	30	25	4.36 ± 0.4
	5	46.2	34	25	4.36 ± 0.4
	6	38.2	34	25	4.04 ± 0.4

[1] The number of metaphase cells per 1000 cells observed was determined five times for each animal and the number appearing in this column represents the average.

[2] Average ± Standard Error of the Mean.

Diesel exhaust did not cause an increase in the frequency of SCE in Chinese hamster bone marrow cells. The B(a)P positive control group responded with an increase in the number of SCE/cell from 4.82 in controls to 10.57 (Table IV). It would appear that diesel exhaust exposure did not result in an increase in sister chromatid exchange in Chinese hamsters even though there was an apparent increase in the number of micronuclei.

TABLE IV. SISTER CHROMATID EXHANGE IN BONE MARROW CELLS OF CHINESE HAMSTERS EXPOSED TO DIESEL EXHAUST (POOLED RESULTS)

TREATMENT	NUMBER OF ANIMALS	MITOTIC INDEX	CELLS OBSERVED	SCE/CELL
CLEAN AIR (CONTROL)	6	39[1]	150	4.82 ± 0.2[2]
B(a)P, 100 mg/kg	4	25	100	10.57 ± 0.5
DIESEL EXHAUST	6	29	150	4.34 ± 0.2

[1] This number represents the average mitotic index obtained by observing 5,000 cells per animal.

[2] Average ± Standard Error of the Mean.

4. Chromosomal Aberration Bioassay

The bone marrow cells were studied for chromosomal aberrations such as chromatid gaps, chromatid breaks, chromosome gaps, chromosome breaks, chromatid deletion, fragmentation, acentric fragments, translocations, triradials, quadriradials, ring chromosomes, dicentric chromosomes, double minutes, polyploidy and aneuploidy. For each animal, 25 metaphase cells were observed (Table V). The number of polyploid cells in both control and diesel treated animals was the same. No aberrations in chromosomes or chromatids were observed in either controls or treated animals. It is apparent that the diesel exhaust does not cause any cytological abnormalities, however, as stated above, there was a decrease in the mitotic index. This investigation is now being extended to study the genotoxic effects of higher concentration of diesel exhaust, diesel particulate matter and its extract.

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TABLE V. POLYPLOIDY AND CHROMOSOMAL ABERRATIONS IN BONE MARROW OF CHINESE HAMSTERS EXPOSED TO DIESEL EXHAUST

ANIMAL NUMBER	TREATMENT	# OF POLYPLOID CELLS	ABERRATIONS* G	B	T	R	F	TOTAL # OF METAPHASES OBSERVED
1	CLEAN AIR (CONTROL)	1	-	-	-	-	-	25
2	CLEAN AIR (CONTROL)	-	-	-	-	-	-	25
3	CLEAN AIR (CONTROL)	1	-	-	-	-	-	25
4	CLEAN AIR (CONTROL)	1	-	-	-	-	-	25
5	CLEAN AIR (CONTROL)	-	-	-	-	-	-	25
6	CLEAN AIR (CONTROL)	-	-	-	-	-	-	25
7	DIESEL EXHAUST	-	-	-	-	-	-	25
8	DIESEL EXHAUST	1	-	-	-	-	-	25
9	DIESEL EXHAUST	-	-	-	-	-	-	25
10	DIESEL EXHAUST	1	-	-	-	-	-	25
11	DIESEL EXHAUST	-	-	-	-	-	-	25
12	DIESEL EXHAUST	1	-	-	-	-	-	25

ABERRATIONS*

G = GAPS

B = BREAKS

T = TRANSLOCATIONS

R = RING FORMATION

F = FRAGMENTATION

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General Discussion

LENBERG: With your sperm morphology testing, it seems to me that the techniques involved were somewhat subjective and you could introduce error just by handling. Was the technician that did the analysis running the operation using the blind analysis method?

P. SABHARWAL: Yes, all these studies are by the blind method. The technicians do not know what samples they are given. They were also scored by the same technicians.

S. KAPLAN: Are some slides submitted to the technician a second time to see if he can replicate the previous reading?

P. SABHARWAL: We have evaluated that variaton by another procedure. Slides that had lready been evaluated were given to us by another laboratory. Our evaluation of the slides agreed with the other laoratory.

S. KAPLAN: Yes, you need reliability as well as validity checks.

J. VOSTAL: In view of the fact that you are stuyding systemic effects and you are using unbelievable high doses, would you be willing to speculate whether much of the observed effects are due to ingestion from grooming and not from inhalation?

A. SABHARWAL: There is always that possibility since the whole animal is placed in the chamber. Even skin absorption could be taking place. This is similar to what happen to us in nature.

J. VOSTAL: I would probably take a very serious exception to your statement. Obviously the exposure route for a person being exposed to concentrations may be ten thousand times lower than those used in the in vivo exposure of animals will be relevant. We are more interested in establishing the relevance of our data to the field situation. We are more interested in finding out the role of potential absorption from a deposit in the respiratory system.

A. SABHARWAL: Would you kindly tell me how you do that?

J. VOSTAL: I suppose that you don't do it unless you are really sure that the dose results in a biological effect. It is not that I would object to the data you can produce by the injection of the diesel particles but it is a question of how shall I interpret those data in view of the question which we all face, namely, will there be any potential artifacts generated by the diesel exposure on our roads?

A. SABHARWAL: I am looking for the same kind of answer so if anybody has an answer we would like to know it.

D. BRUSICK: Were the sperm abnormalities also done in hamsters or just the mosue?

A. SABHARWAL: All the work reprted was in Chinese hamsters. A report on sperm abnormalities is reported in another presentation at this symposium.

D. BRUSICK: Since the effects observed in sperm abnormalities were significant, but not greatly different than controls, do you have a reasonable historical data base in this species which would indicate the sort of variance and background that you would expect.

A. SABHARWAL: We need to perform more experiments before we answer that question.

SISTER CHROMATID EXCHANGE ANALYSIS OF SYRIAN HAMSTER LUNG CELLS TREATED IN VIVO WITH DIESEL EXHAUST PARTICULATES

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Abstract

Polycyclic aromatic hydrocarbons extracted and concentrated from diesel exhaust particulates have been shown to be mutagenic and carcinogenic, but attempts to induce pulmonary tumors through chronic inhalation of diesel exhaust by experimental animals have failed. We have attempted to resolve this incongruity by measuring chromosomal damage in lung tissue of chronically exposed hamsters, using the highly sensitive test for chemical mutagens, sister chromatid exchange (SCE) analysis.

To determine the degree of responsiveness of the test system to both diesel exhaust particulates and benzo(a)pyrene (BaP), these agents were instilled intratracheally into anesthetized hamsters as suspensions in 0.25 ml volumes of Hank's balanced salt solution (HBSS). Lung tissues from these animals were subsequently cultured _in vitro_ and chromosomes from the resulting cell divisions were scored for exchanges of chromatin between sister chromatids.

Control animals, treated weekly with 0.25 ml of BSS for 10 weeks, showed an average value of 12 SCE's per cell, while animals treated weekly with 200 ng BaP over a 10-week period showed an average of 17 SCE's per cell. HBSS, given as a single treatment also produced an average of 12 SCE's per cell in control animals, but animals treated with a single instil-

lation of 12.5 ug BaP showed an average SCE value of 19. These data confirmed that the procarcinogen BaP can be metabolically activated by lung cells in vivo and also demonstrated the efficacy of using this technical approach to study the effect of chemical mutagens that enter the lungs.

Diesel exhaust particulates, administered in a range from 0 to 20 mg per hamster over a 24-hour exposure period, produced a linear SCE dose-response ranging from 12 to 26 SCE's per metaphase. This curve suggested that a concentration of 3 mg of diesel particulates per hamster would not produce a statistically significant increase in SCE's above control values. One group of 8 hamsters, chronically exposed to diesel exhaust particulates for 3 months showed an average of 12 SCE's per cell. This was equivalent to a set of 5 control animals which also showed an average of 12 SCE's per cell.

Although the scope of this study was limited, the data demonstrated that a 3-month exposure to 6 mg/m^3 of diesel exhaust particulates was insufficient to produce measurable mutagenic changes in lung cells. This negative response is consistent with the results from other studies in which similar exposures failed to produce pulmonary tumors.

INTRODUCTION

Diesel exhaust contains a wide variety of polycyclic aromatic hydrocarbons (PAH) adsorbed onto carbon particulates(1). These agents, when extracted and concentrated, have been shown to be mutagenic in the Ames test(2) and carcinogenic when painted on the skin of C57 Black and A strain mice(3). However, chronic exposure studies at concentrations and exposure times which produce fibrosis and emphysema in experimental animals, have failed to induce tumors in the respiratory tracts of these animals(4).

Development of a mutagenesis/carcinogenesis response requires an exposure to appropriate concentrations of carcinogenic agents over suitable lengths of time. Since chronic exposure studies have been conducted for half the lifetime of the experimental animals and have caused respiratory pathology other than tumor induction(4), it is most probable that the concentration of carcinogens in diesel exhaust have been too low to produce the carcinogenic response, even when diesel engine performance was adjusted to maximize the production of PAH. An extrapolation of the dose-response curve, published by Saffiotti, et al(5) showing a linear relationship between benzo(a)pyrene (BaP) concentration and lung tumor formation in hamsters, suggests that a minimum of 2.5 mg BaP, accumulated over a 30-week exposure period, would be necessary to produce a significant number of lung tumors. However, in diesel exhaust studies with hamsters, one can calculate with respiratory total flow data that if all carbon particles from a chronic exposure (10 mg particulates/m^3 for 5 hrs/day, 5 days/week for 1 year) were retained in the lung, hamsters would accumulate no more than 50 mg of particulates. Huisingh, et al(2) have reported that diesel exhaust can contain up to 25 ng BaP/mg of particulate. Therefore, the maximum BaP exposure per animal would be 1.25 ug or about 2,000 times less than the concentration necessary to cause lung cancer. Additional factors such as plating of particles on the nasomucosal membranes(6), mucociliary clearance and inefficient elution of the carcinogens from the particulates by lung tissue fluids(7) would make the differential even more extreme. The accuracy of this extrapolation can be tested by performing sister chromatid exchange (SCE) analysis on primary cultures of hamster lung cells following *in vivo* exposure to diesel exhaust or any diesel constituent. SCE analysis is a very sensitive short-term bioassay for measuring genetic damage caused by mutagens/carcinogens(8). SCE analysis can detect mutagenic changes following exposure to nanogram or microgram concentrations of mutagen(9).

Polycyclic aromatic hydrocarbons eluted from inhaled carbon particles can be metabolically activated by aryl hydrocarbon hydroxylase contained in tracheal(10) and bronchial tissues(11). The active products bind with cellular DNA(12) and the resultant genetic alteration can then be visualized as an increase in SCE's(13). This experimental approach, though technically difficult, is superior to other systems for assessing the actual mutagenic risk incurred by animals following *in vivo* exposure to diesel exhaust and other chemicals with *low* mutagenic potential, due to the inherent sensitivity in the system.

MATERIALS AND METHODS

Preparation of the Diesel Exhaust Particulates. The particulate fraction from diesel exhaust was collected on Gelman A/E glass fiber filters by personnel in the Health Effects Research Laboratory, Cincinnati, Ohio, then frozen and shipped to our facility in Pasadena, California, for testing. The particulate fraction was brushed from the filters with a camel's hair brush. This process produced a heavy contamination with glass filter fibers. Therefore, the diesel particulate-fiber mixture was suspended in 2% Emulphor EL-620 (GAF Corp., New York, N.Y.), a nonionic wetting agent. This allowed the particles to dissociate from the fibers and made it possible to separate the two components with differential centrifugation (5 minutes at 1000 rpm in a clinical centrifuge). The supernatant, containing the particulate suspension, was collected and pooled from 15-20 washings of the glass fiber pellet. A residual contamination of short glass fibers was removed by filtering the pooled supernatant through number one Whatman filter paper.

The carbon particles were concentrated by centrifugation at 10,000 rpm for 30 min. in a refrigerated International centrifuge. The pellet was then washed twice with distilled water to remove the Emulphor. The pelleted particulates were spread on the floor of glass 100 mm diameter petri dishes and air dried in a dessicator. The dried residue was scraped off the glass with a stainless steel spatula, then was weighed on an analytical balance and resuspended in Hank's balanced salt solution just prior to intratracheal instillation.

Preparation of Benzpyrene/Hematite Suspensions. Equal weights of BaP (Sigma Chemical Co., St. Louis, Missouri) and hematite (Fisher Scientific Co., Philadelphia, Pennsylvania) were placed in a mortar and ground together for an hour. This preparation gave a fine, homogeneously distributed dust containing 50% BaP and 50% hematite by weight. In preparing the dust, care was taken to ensure that both samples were dry to avoid formation of large clumps. The dust was stored at 0° C in aluminum foil wrapped tubes to exclude light. Just prior to use, the dust was weighed on an analytical balance and dispersed in the BSS by using ultrasound and a vortex mixer alternately for 30 minutes.

Treatment of Experimental Animals. The experimental animals used in the intratracheal instillation portion of this study were 4-6 week old male Syrian hamsters obtained from Simonsen Laboratories, Inc., Gilroy, California. A separate group of control and experimental animals were chronically exposed to diesel exhaust in Cincinnati, Ohio. The animals were exposed in environmental chambers supplied with clean air or with air containing 6.39 ± 0.78 mg/m^3 diesel exhaust particulate (DEP) for 8 hrs/day, 7 days/week for a period of about 3 months (Table 1). The animals were flown to our laboratory at the end of the 3 months for SCE analysis.

The intratracheally treated animals were given 0.25 ml volumes of either HBSS solution, DEP suspended in HBSS or BaP/hematite suspended in HBSS, using the intratracheal instillation procedure described by Saffiotti, et al.(14). The animals were anesthetized with an intraperitoneal injection of 6.7 ml/kg of a 1% solution of sodium brevitol (Eli Lilly & co., Indianapolis, Indiana). Each anesthetized animal was placed on a slanted board. With its back on the board, its mouth was kept open by hanging the lower incisors on a wire hook while the

Table 1

Component Concentrations in Exposure Chamber

Components Monitored	Reference Chambers	Exhaust Emission Chambers
CO_2, %	0.05 ± 0.01	0.29 ± 0.03
CO, ppm	2.07 ± 0.53	19.72 ± 2.13
HC, ppm as C	3.49 ± 0.38	7.84 ± 0.99
NO, ppm	0.08 ± 0.03	11.23 ± 1.53
NO_2, ppm	0.05 ± 0.03	2.65 ± 0.55
SO_2, ppm	0.05 ± 0.01	2.06 ± 0.43
Particulate, mg/m^3	-	6.39 ± 0.78

upper incisors were retained by a tight rubber band. All of the solutions and particulate suspensions were maintained at 37° C in a water bath. Just prior to instillation, each suspension was vortex mixed for 5 seconds then was drawn in and out of a 1.0 ml tuberculin syringe fitted with a blunt 19 gauge needle several times to further insure uniform distribution of the particulates, then the syringe was filled with 0.25 ml of the test suspension. The blunt needle was about 60 mm long and bent at a 135° angle 45 mm from the tip. A direct focusing lamp from a dissecting microscope provided a view of the pharynx after the tongue of the hamster was gently pulled outward and laterally with forceps. The tip of the needle was inserted under the epiglottis to uncover the vocal cords, then gently inserted into the trachea. The needle was pushed almost to the bottom of the trachea, then the suspension was gently injected and the needle withdrawn. Following instillation of the suspension into the lungs, the animals showed a brief apnea, after which they rapidly recovered and resumed regular respiration.

Tissues Culture Procedure. The animals were sacrificed by dislocation of the cervical vertebrae and then heart and lungs were quickly excised and transferred into sterile 100 mm petri dishes. The heart, trachea and bronchi were removed and the remaining lung tissue was finely minced with sterile scissors in 3 ml McCoy's 5A medium supplemented with 10% fetal calf serum, 100 units of penicillin, and 100 ug streptomycin/ml. The dishes were allowed to incubate overnight in a 5% CO_2 incubator at 37° C. The following day unattached cells and tissue fragments were removed, washed in NBSS to remove erythrocytes and cellular debris, then the viable cells and tissue fragments were distributed into 3 additional petri dishes for each lung. The attached cells in the original dish were washed once with HBSS, to remove cellular debris, then the HBSS was replaced with complete McCoy's medium supplemented with 10% fetal bovine serum.

All four culture dishes for each animal were incubated in a CO_2 incubator at 37° C. The earliest mitotic figures were observed at about 60 hrs of incubation and colonies of 50-200 cells were formed at about 5-8 days (Fig. 1). Cultures containing lung tissue previously treated with either benzypyrene or low concentrations of DEP were often seen to progress faster than cultures of control lung. However, cultures from animals given a total of 100 mg DAP over a span of 10 weeks showed cells which were engorged with DEP (Fig. 2). These cultures showed no mitotic activity over several weeks of incubation.

Sister Chromatid Exchange Analysis. When the cultures showed colonies containing 50 or more cells, they were treated with

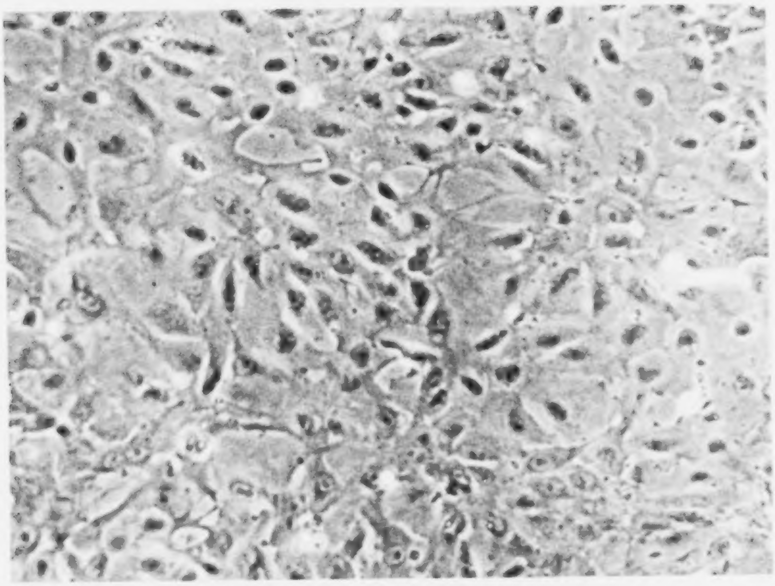

Figure 1. Photomicrograph of epithelial cells from 8-day culture of Syrian hamster lung tissue.

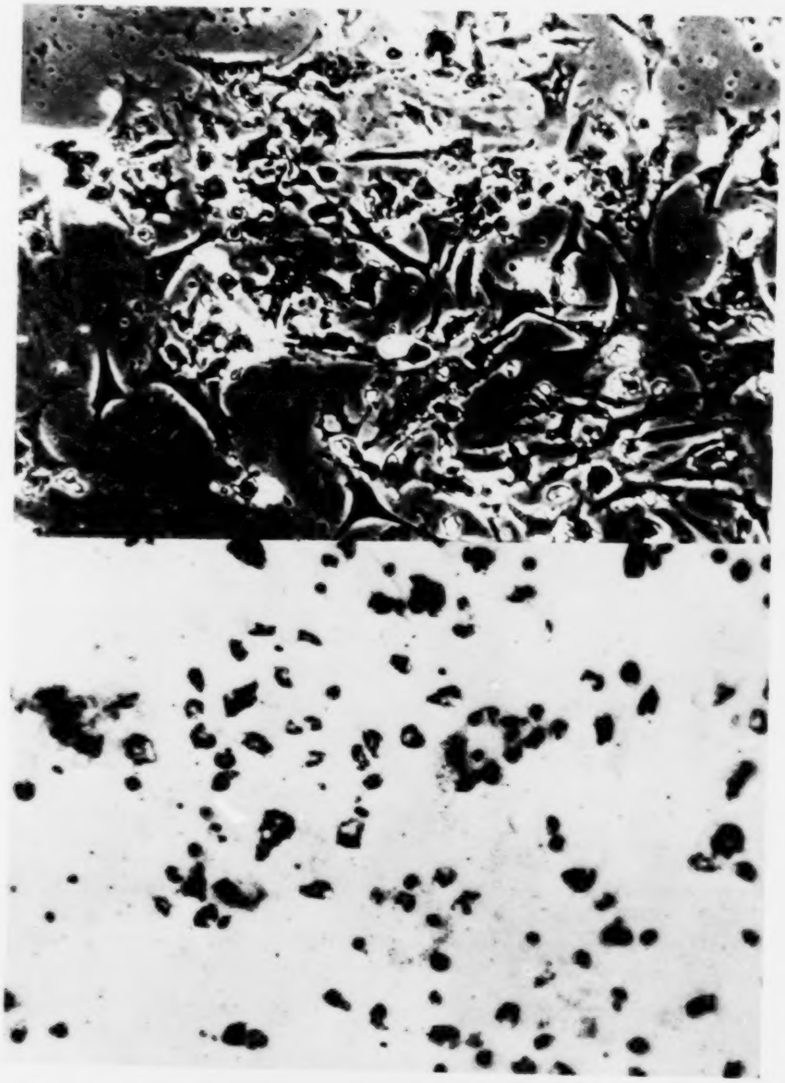

Figure 2. Carbon laden lung cells from a hamster which had received 10 mg DEP/week for 10 weeks. Top, Phase contrast image showing cellular detail. Bottom, Bright field image of the same field showing opaque diesel particles in the cytoplasm.

10 ug bromodeoxyuridine (BrdU) in subdued illumination. The dishes were wrapped in aluminum foil and incubated in the dark for an additional 40 hrs, during which time the cells completed 2 cell cycles. The cultures were then treated with 0.05 ug/ml colcemid (Grand Island Biological Co.) for 3 hrs to block cell division and accumulate as many mitotic figures as possible. The cells were harvested with trypsin (0.05% trypsin in Ca^{++}, MG^{++} Free HBSS) and treated for 30 minutes at 37° C in 0.075 M KCl. The cells were then fixed in cold (4° C) Carnoy's solution (1 part glacial acetic acid: 3 parts methanol), washed 2 times in cold Carnoy's solution and dropped on clean glass microscope slides to form chromosome spreads.

When the slide preparations were thoroughly dry, they were stained for 20 minutes at room temperature in a 5 ug/ml solution of Hoechst 33258 (American Hoechst Corp., Somerville, New Jersey) in 1/15 M Sorensen's buffer (pH 6.8). The slides were placed in a pyrex baking tray and covered with 1/15 M Sorensen's buffer. The buffer was overlayed with Saran wrap to minimize water evaporation. Then the slides were exposed to light from a bank of Gro-lights (General Electric Co., Cleveland, Ohio) overnight (18 hrs). The cells were then stained with freshly prepared 3% Giemsa (Scientific Products, Irvine, California) in 1/15 M Sorensen's buffer (pH 6.8). The sister chromatid exchange frequency was scored in 20-30 spreads for each animal using oil immersion bright field optics.

RESULTS AND DISCUSSION

Appraisal of the Test System. Preliminary studies with Lab-Tek culture vessels (Miles Laboratories, Inc., Naperville, Illinois) demonstrated that chromosome spreads, suitable for SCE analysis, could be obtained after 4-5 days of culture (Fig. 3). However the yield of these spreads was low, especially for treated lung tissue. Therefore, we elected to harvest cells after 8-10 days of culture. It is not likely that the SCE frequency was reduced due to DNA repair during this incubation period because the DEP treated lung cells contained visible carbon particle inclusions (Fig. 4) which persisted throughout the culture period. Therefore, the cells were essentially subjected to a continuous DEP treatment until they were fixed and stained for SCE analysis.

Mean SCE values for 12 control animals are shown in Table 2 and Fig. 5. These mean values ranged from 9.3 to 14.1 and showed an average of 11.8 for the 12 animals. By way of comparison, 4 animals sacrificed 24 hrs after single instillations of 12.5 ug BaP, showed a range from 18.0 to 20.7 SCE's, with an average of 19.5. Five positive control animals given weekly instil-

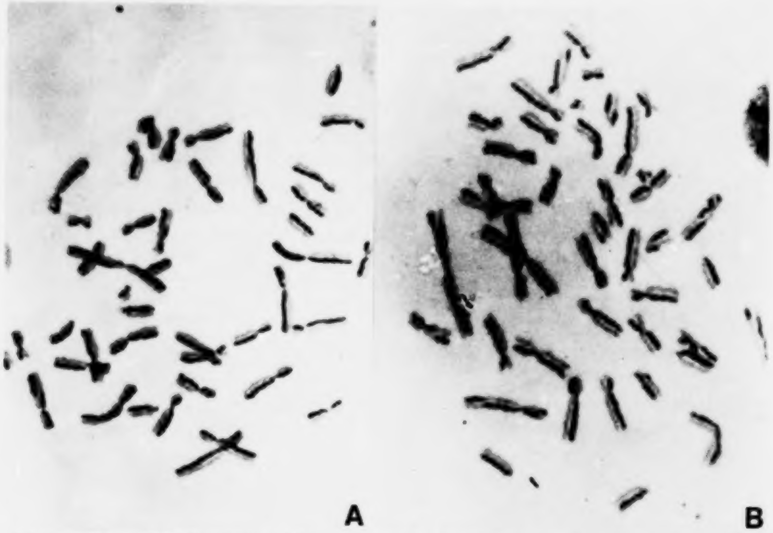

Figure 3. Differentially stained chromosome spreads from 96 hr. cultures of hamster lung. A. Spread from a control hamster showing few SCE's. B. Spread from a BaP treated hamster showing several SCE's.

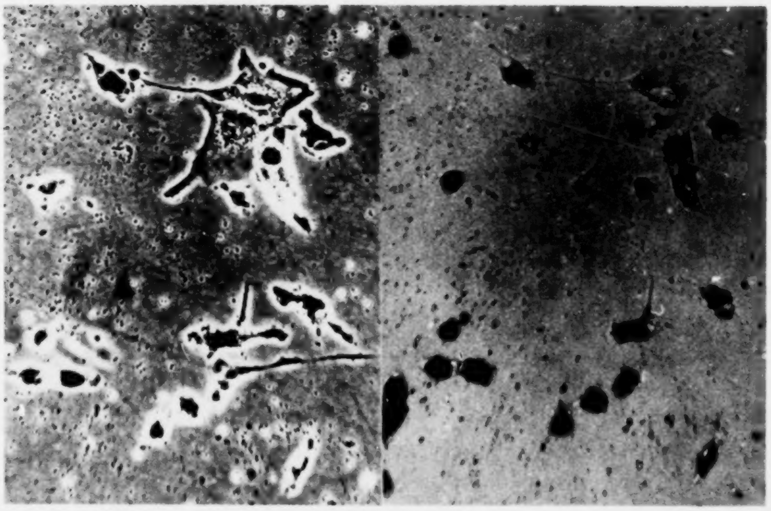

Figure 4. Epithelial cells from a 7-day hamster lung culture. Left, Phase contrast image showing cellular detail. Right, Bright field image showing carbon particle inclusions in the cytoplasm.

Table 2

Mean SCE Values for Control and BaP Treated Hamsters

Control Animals	Benzo(a)pyrene treated animals 1 x 12.5 ug	10 x 0.2 ug
10.1	18.0	-
13.1	19.9	-
11.1	19.4	-
12.3	20.7	-
9.3	-	-
11.4	-	-
13.7	-	16.3
12.0	-	16.5
11.9	-	18.7
11.9	-	16.8
11.1	-	17.4
14.1	-	-
11.83 ± 0.42*	19.50 ± 0.65*	17.14 ± 0.48*

*Average SCE values ± SE for the sets.

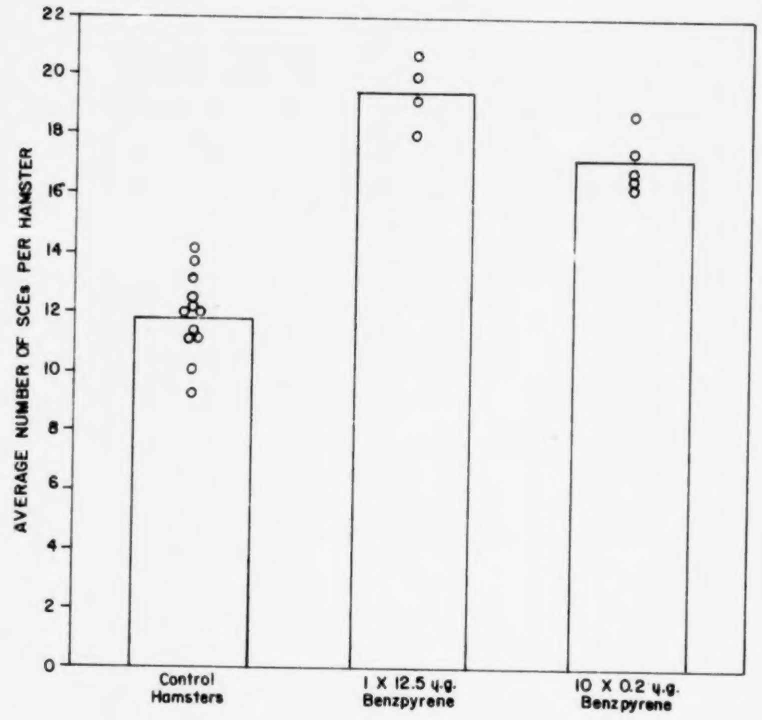

Figure 5. Histogram of the SCE frequency in control and BaP treated hamster lung tissues. The circles represent the mean SCE values for each animal in the set. The height of the bar represents the average value for each set.

lations of 200 ng BaP per week for 10 weeks had an average of 17.1 SCE's with a SCE range of 16.3 to 18.7. Although these data are not optimized for maximum responses (i.e., activation times vs DNA repair rates) they demonstrate (a) that it is technically feasible to perform SCE analysis on primary cultures from control and treated hamster lungs, and (b) BaP can be metabolically activated in vivo(10,11), and can produce a significant increase in SCE's above control values. The accumulated dose of BaP over the 10-week interval was only 2 ug. This sensitivity level appears to be 3 orders of magnitude greater than pulmonary tumor production in Syrian hamsters intratracheally instilled with milligram quantities of BaP(5).

Dose-Response Curve for DEP. Primary lung cultures can be unsuitable for SCE analysis for several reasons: (a) harvested cells can fail to propagate, (b) mitotic figures can be lost during the chromosome spreading procedure, (c) the spreads that are found can be either in the first or third cell cycle and therefore are not suitable for scoring, and (d) lungs treated with relatively high DEP concentrations yield spreads with so much carbon that the chromosomes are obscured (Fig. 6) which makes SCE scoring unreliable or impossible. Because of these factors, we were only able to collect adequate data from one hamster for each of the DEP concentrations tested (0, 6, 11 and 20 mg of DEP). The mean SCE values and the standard errors of the means for these 4 animals were 11.4 + 0.2, 16.7 + 0.1, 19.8 + 0.1 and 26.4 + 0.3, respectively. Although these data form a linear dose-response curve (Fig. 7), caution should be exercised in interpreting its significance. We can conclude, however, that the primary lung cells do respond to DEP as a mutagen over a 24 hr treatment period.

Chronic Exposures to DEP in the Atmosphere. Lung cell cultures provided suitable numbers of differentially stained chromosome spreads for 5 control animals. The range of mean SCE values for these hamsters was 10.1 to 13.1 with an average of 11.52 + 0.58 (Table 3). Eight animals chronically exposed to 6.39 mg DEP/mg^3 for 3 months gave suitable cultures for analysis. The range for this set was from 10.0 ot 14.8, with an average of 11.86 + 0.47.

Paintal(15) has calculated that a 92 g Syrian hamster inhales an average of 0.06 liters of air per minute. Using this value, we can calculate that animals given a chronic exposure of 6 ug DEP/m^3, 8 hrs/day, 7 days/week, for 3 months, would accumulate a maximum of 15.6 mg of DEP. However, losses due to DEP plating on the nasal mucosa, mucociliary clearance, and pulmonary macrophase phagocytosis would reduce the DEP concentration to an estimated 1.5 to 3.0 mg/animal. The preliminary dose-response data in Fig. 7 suggest that no significant increase in SCE's would be expected for this level

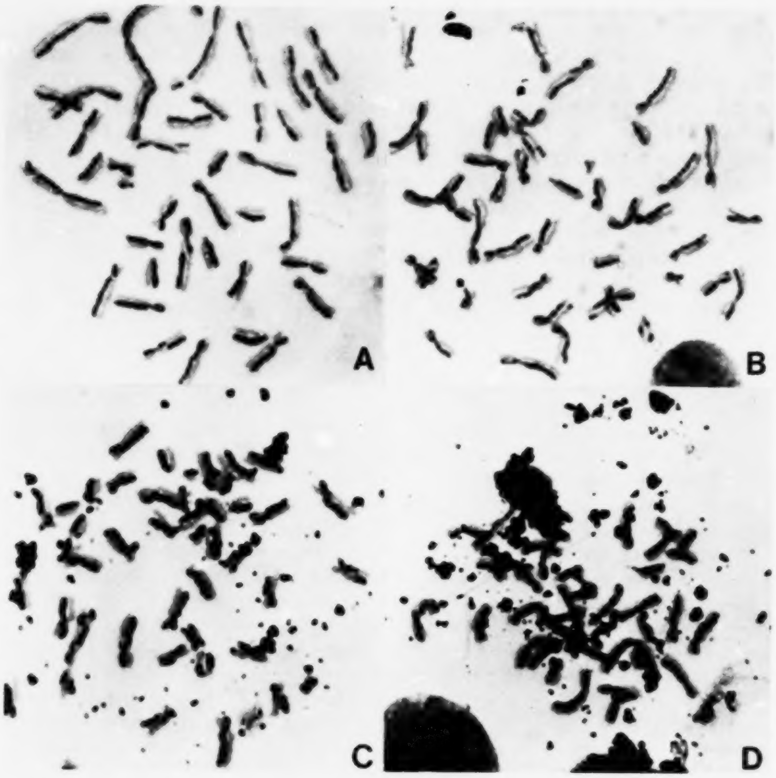

Figure 6. Differentially stained chromosome spreads showing varying amounts of carbon particulate inclusions. A. Spread from a control animal. B. Spread from an animal receiving 6 mg DEP. C. Spread from an animal receiving 11 mg DEP. D. Spread from an animal receiving 20 mg DEP.

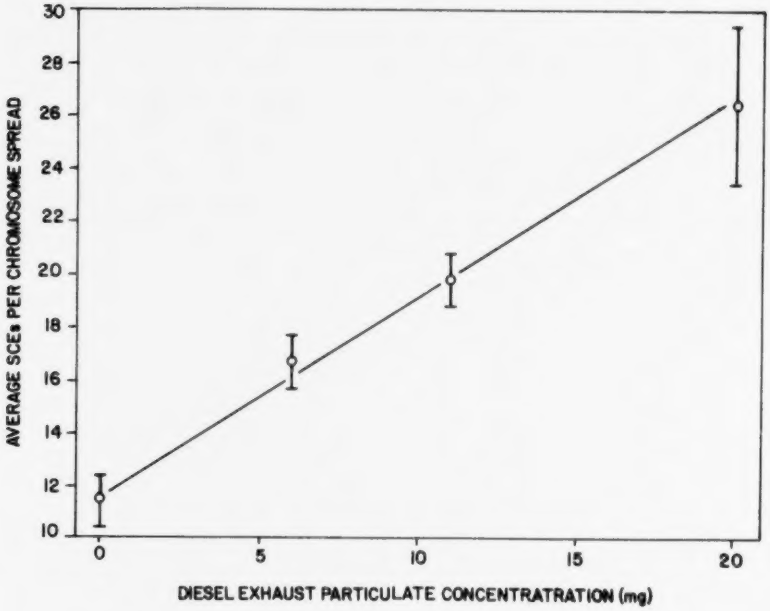

Figure 7. Dose-response curve of the SCE frequency resulting from DEP administered *in vivo* over a range from 0 to 20 mg per hamster by intratracheal instillation.

Table 3

Mean SCE Values for Hamsters Exposed for Three Months to Control Air or Diesel Exhaust Emissions

Control Air	Diesel Exhaust
10.1	11.6
13.1	14.8
-	10.9
-	11.9
11.1	12.2
-	10.0
12.3	11.1
11.0	12.4
11.52 ± 0.58*	11.86 ± 0.47*

*Average SCE value ± SE for the sets.

of DEP exposure. The data in Table 3 support that prediction. It is reasonable to assume that if the sensitive SCE test shows measurable response following 3 months chronic exposure, pulmonary tumor production would be highly unlikely(4).

Since SCE increases were induced with intratracheal instillation of DEP (Fig. 7), we must assume that longer exposures and higher concentrations of DEP would show a statistically signficiant increase in the SCE response. Such data would provide an important basis from which risk assessments for DEP exposures could be made.

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General Discussion

D. BRUSICK: In one of the preceeding paper we saw some data using extracts in Chinese hamster cells in vitro which indicate that sister chromatid exchange could be induced. Now we have data in hamsters, for both the particles and extracts. Do you think that the dose received in vivo can account for the difference observed under in vitro situation versus what we observed in vivo?

D. ROUNDS: Basically, I believe it is a matter of dose but we have to consider that the whole animal is a very complex system. We have to think about the method by which acting components can be diluted off the carbon and distributed throughout the system.

D. BRUSICK: It might be interesting, and maybe not even too far fetched, to consider an experiment of the type that you have done with a combination of diesel inhalation exposure at low concentration and other environmental materials such as benzene. In other words, the combination of the particulates and solvents be worth looking at?

D. ROUNDS: Yes, we had hoped that we could use the technique for any kind of air pollutant. We have previously done some studies with gene cells which were derived from the human body when exposed to ozone in vitro sister chromatid exchange was induced. This would be a good way to examine ozones, benzene, or any kind of ir pollutant in order to evaluate the kind of erosol and the kind of mutagenic responses that can occur in te pulmonary tract of the exposed animal. S. SODERHOLM

S. SODERHOLM: I don't believe you mentioned in your calculation of the inhaled dose a deposition efficiency. There is actually an experimental number that says a deposition efficiency in the deep lung, meaning that which was retained after 24 hours, is roughly 20 percent for the size of particles used in your experiments. Was that taken into account?

D. ROUNDS: We estimated 10 to 20 percent for the one and a half to three milligrams that I quoted. Three millograms would result from a 20 percent deposition efficiency.

D. NETTESHIM: I believe it would be wise in the future, in such experiments, to incorporate some controls with some other dust. I wonder if by instilling some innocusus material such as ferric oxide, whether a similar SCE response would be obtained? It might also be worthwhile to look at the lymphocytes in the lymph nodes, since the material is going to be trapped there for a long time. I don't know whether there are enough cells there for you to use the SCE test, but I think this is another possibility to consider.

D. ROUNDS: We had thought about using a powder of activated charcoal for the intratracheal instillation but described against it because there are all kinds of organic material absorbed to charcoal. The lymphocyte approach could be a very logical extension to the SCE test.

TEST OF DIESEL EXHAUST EMISSIONS IN THE RAT LIVER FOCI ASSAY

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ABSTRACT

An initiation/promotion assay in rat liver has been developed by Sells et al (1). Partial hepatectomy was used to enhance initiation, and a choline-devoid diet as promoter. The induction of carcinogenesis was determined by the focal appearance of gamma glutamyl transpeptidase (GGT) positive hepatocytes. We adopted this assay to diesel exhaust emission by performing a 2/3 partial hepatectomy, and exposing the rats to either clean air or diesel exhaust emission. The rats were fed either a choline-devoid or a choline-supplemented diet for three or six months. The animals were sacrificed and liver sections stained for GGT were examined for the presence of foci of GGT(+) hepatocytes. The results indicate that diesel exhaust exposure does not result in a systemic dose of carcinogens sufficient to be detected in the liver foci assay.

INTRODUCTION

Diesel exhaust emissions contain thoudands of organic chemicals, some of which are carcinogenic polycyclic aromatic hydrocarbons. The high particle concentration of diesel exhaust results in sequestration of much of the polycyclic aromatic hydrocarbons in the particles. The sequestered carcinogens must be eluted in order to be active. Therefore, the assessment of the health hazard of exposure to diesel exhaust requires the demonstration in vivo of its carcinogenic activity in a bioassay impacted by pharmacokinetic and metabolic factors unique to particle-bound carcinogens.

The two stage evolution of carcinogenesis, consisting of an initiation and a promotion stage, originally described by Berenblum for mouse skin (2, 3) has been demonstrated in many other tissues (4-8). Promoters are agents that reduce the latent period required for the appearance of tumors, but have no ability to initiate target cells. Tumor promoters have been described for skin, liver, lung, bladder and colon carcinogenesis (4-9).

Initiation/promotion bioassays are being developed, for testing the potential carcinogenicity of envioronmental factors, that take advantage of the reduction in the time required for the appearance of either preneoplastic lesions or of tumors afforded by the use of promoters. Partial hepatectomy has been shown to enhance the initiation of rat liver cells by chemical carcinogens (10-11). Thus, administration of non-liver carcinogens, including 7-12- dimethylbenz(a)anthracene (12), within twenty-four hours after a partial hepatectomy, results in liver cancer. Initiated liver cells can be detected, histochemically, from the development of foci of GGT(+) hepatocytes (13-14), and the evolution of initiated cells to foci of GGT(+) hepatocytes is efficiently promoted in rats fed a choline-devoid (CD) diet (15). Therefore, the bioassay in rats outlined in figure 1 involves 1) A 2/3 partial hepatectomy, to enhance initiation; 2) feeding a CD diet as a promoter; and 3) induction of foci of GGT(+) hepatocytes as the end point. Using this bioassay, we have attempted to assess the carcinogenicity of diesel exhaust.

MATERIALS AND METHODS

Male Sprague-Dawley rats (Charles River), weighing 150-175 gm, were used. The choline supplemented (CS) and CD diets were prepared as described by Shinozuka et al (15). The rats received a 2/3 partial hepatectomy or a sham operation, and exposure to either clean air or diesel exhaust was begun on the same day. The rats were fed the CS or CD diet ad libitum.

The diesel engine and exposure conditions at the U.S. Environmental Protection Agency, Health Effects Research Laboratory facilities in Cincinnati, Ohio, are described by Hinners (16). Briefly, the six cylinder Nissan engine used was run on the Federal Short Cycle. The animals were exposed to diesel exhaust for eight hours daily at 6 mg/m^3 particles (1:18 dilution).

After three and six months of exposure, and feeding either the control CS diet or the CD diet, groups of rats were sacrificed by decapitation and the liver excised. Small blocks of liver tissue were fixed in 95% ethanol - 1% acetic acid, dehydrated and embedded in paraffin. Sections (8 um) were stained for gamma glutamyl transpeptidase activity by the method of Rutenberg et al (17) or with hematoxylin-eosin. Approximately 2-3 cm^2 of liver sections from each rat were examined for the presence of foci of GGT(+) hepatocytes (Figure 1).

RESULTS AND DISCUSSION

The rats were divided into six groups of 25 each and were treated as described in Table 1. Table 1 contains also the experimental results. Significant numbers of foci of GGT(+) hepatocytes developed in none of the groups of rats, in particular in those exposed to diesel exhaust for three or six months. There was also no remarkable liver toxicity after six months of exposure. It is evident, therefore, that the bioassay used in these studies, is unable to reveal the carcinogens and hepatotoxins which are present in diesel exhaust. This inability could be due to: 1) the sequestration of the carcinogens and hepatoxins in the diesel exhaust particles, so that they are not distributed systemically; and 2) the rat liver foci bioassay, as employed in these studies, is not sensitive enough.

ACKNOWLEDGEMENT

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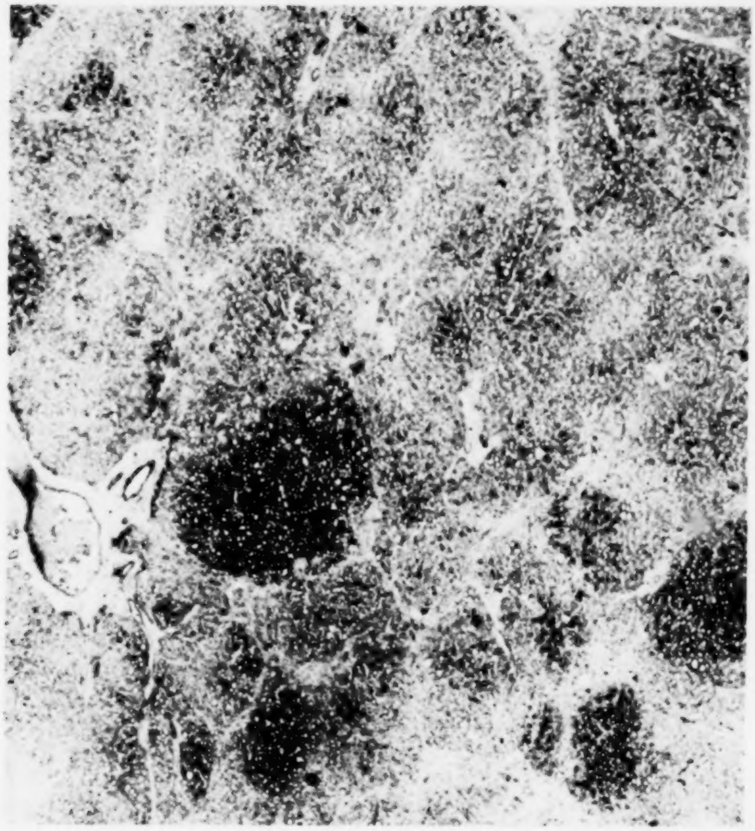

Figure 1. Foci of gamma glutamyl transpeptidase positive hepatocytes. Rats were administered 100 mg/kg diethylnitrosamine by intraperitoneal injection. The rats were fed laboratory chow for a week and then fed a choline-devoid diet for six weeks. The dark areas are GGTase positive islands (70X).

Table 1. Rat Liver Bioassay of Diesel Exhaust

Treatment	No. of GGT(+) Foci/cm^2[a]
1. P.H.[b] - Diesel Exhaust - CD diet	
3 months	0
6 months	0.20 ± 0.20
2. P.H. - Diesel Exhaust - CS diet	
3 months	0
6 months	0
3. S.O.[c] - Diesel Exhaust CD diet	
3 months	0
6 months	2.28 ± 2.28
4. P.H. - Clean Air - CD diet	
3 months	0
6 months	5.68 ± 3.07
5. P.H. - Clean Air - CS diet	
3 months	0
6 months	0.29 ± 0.29
6. S.O. - Clean Air - CD diet	
3 months	0
6 months	2.82 ± 1.91

a - At least 2 square centimeters were examined for the presence of GGT(+) foci.

b - P.H. = 2/3 partial hepatectomy.

c - S.O. = Sham Operation

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THE EFFECT OF DIESEL EXHAUST ON SPERM-SHAPE ABNORMALITIES IN MICE

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ABSTRACT

The sperm-shape abnormality bioassay in mice was used to determine whether chemical mutagens in diesel exhaust reach the testes. Strain A male mice (30 per group from 4 to 6 weeks of age) were exposed for 31 or 39 weeks to either diesel exhaust or clean air. After exposure, Eosin y-stained, air-dried smears of cauda epididymal sperm were scored for changes in sperm-head abnormalities in three different laboratories. There was no difference in the proportion of abnormally shaped sperm in controls and mice exposed to diesel exhaust.

Diesel exhaust emissions contain thosuands of organic chemicals, including known carcinogens and mutagens. Because of the high particle concentration of diesel exhaust a large proportion of the organics, especially the polycyclic aromatic hydrocarbons, are adsorbed onto particles (1). For genotoxic agents in diesel exhaust to affect the mammalian testes they must be deposited in the lungs, eluted from the particles, and distributed in the body so that active metabolites can reach the testes.

The murine sperm-shape abnormality bioassay provides a way of assessing possible spermatoxic and genotoxic effects of chemical agents *in vivo* (2). Studies in mice have shown that a) sper-head shape is under genetic control, b) chemical induction of sperm abnormalities is correlated with germ-cell mutagenicity, and c) mutations associated with induced sperm-abnormalities can be inherited in the offspring of treated mice (3). Several other factors, such as x-rays, heat, and severe fevers involving the testes can also induce sperm-head abnormalities (3). Besides identifying testicular toxins and mutagens the sperm abnormality assay may also be an effective indicator for carcinogens. For example, sixty-five (65%) percent of 38 known carcinogens tested by this bioassay assay showed positive results, a correlation very similar to that found using the *Salmonella* bioassay, which is associated with carcinogenic potential (4).

In this communication, we report the effect of inhalation exposure to diesel exhaust on the murine testes using the sperm-head abnormality assay.

METHODS AND MATERIALS

Male mice (strain A/Strong, Strong Research Foundation, Calif.) at 4 to 6 weeks of age were divided into 4 groups of 30 mice each and exposed to either clean air or diesel exhaust emissions for 31 or 39 weeks. Thirty-one and 39 weeks were chosen because they represent approximately 6 and 8 complete spermatogenic durations. This experiment design permitted 2 independent assessments of the effects of diesel. The diesel exhaust engine and exposure conditions at the U.S. Environmental Protection Agency, Health Effects Research Laboratory facilities in Cincinnati, Ohio, are described elsewhere by Hinners (5). Briefly, a 6-cylinder Nissan diesel engine was run on the Federal Short Cycle and animals were exposed to exhaust for 8 hours daily at 6 mg/m^3 particles concentration using a 1:18 dilution.

At the end of the exposure interval, the mice were sacrified by cervical dislocation, the cauda epididymides excised, and minced in 4 ml of phosphate buffered physiological saline. The suspension from each mouse was mixed by repeated pipetting and filtered through 74 μm nylon mesh. An aliquot was mixed (10:1) with 1% Eosin-Y prepared in water and incubated for 30 min. Smears were made, air-dried, and mounted with Permount. For each mouse in the control and exposed groups receiving 31 week exposures, 500 sperm were examined in each of three different laboratories. The control and exposed mice receiving 39 week exposures were examined at only one of these laboratories. Examples of normal and abnormal sperm shapes are presented in Figure 1.

RESULTS AND DISCUSSION

The effect of diesel exhaust emission on sperm shape is summarized in Table 1. Although there were some differences in the scoring criteria used in the 3 labroatories, none of the laboratories detected a difference in the percentage of abnormally shaped sperm in mice exposed for 31 weeks to diesel exhaust when compared to clean air controls. Laboratory III scored an additional 2 groups of 30 mice each, that were exposed for 39 weeks to either clean air or diesel exhaust. As shown in Table I, the percentages obtained for these animals were not statistically different from the values obtained for the 31 week exposure, and also showed no difference between control and exposed mice.

These results show that inhalation exposure to diesel exhaust did not induce a detectable increase in sperm head abnormalities in mice used. The failre to detect an increase suggests that the testes is not affected by inhalation of diesel exhaust. The negative finding, however, may be due in part to the genotype used in this study. Although the A/Strong strain is genetically able to activate and metablize hydrocarbons, it has a very high spontaneous rate of sperm shape abnormalities in unexposed males (approximately 27%, see Lab III, Table 1) when compared to the 1 to 2% found in B6C3F1 hybrid males which are normally used in this bioassay (6). This high background rate and the unusually high variance in the unexposed mice could have masked any small positive effect. The failure to detect increases in sperm shape abnormalities might also have been due to 1) the low levels of genotoxic material sin diesel exhaust, 2) the strong adsorption of such chemicals onto the diesel particles which may have prohibited their release in vivo, or 3) the inability of the released materials to be transported from the lung, to be metabolically activated, or to reach the testes.

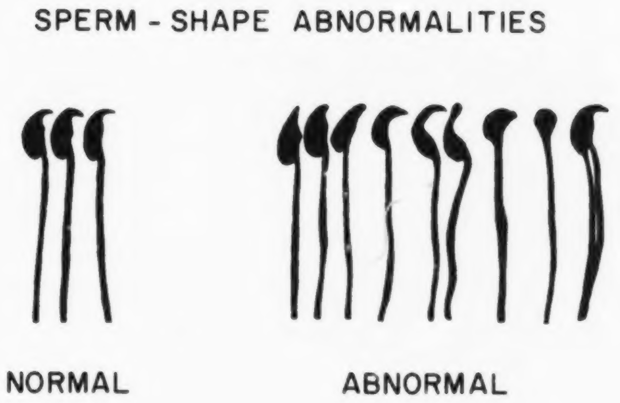

Figure 1. Shapes of normal and abnormal sperm from mice.

TABLE I. EFFECT OF DIESEL EXHAUST ON SPERM MORPHOLOGY IN MICE

Treatment	Number of Animals	Percentage of Abnormally Shaped Sperm			
		Lab I[a]	Lab II[a]	Lab III[a]	Lab III[b]
Diesel Exhaust	30	12.0 ± 7.7[c]	13.9 ± 7.5	27.7 ± 9.7	27.1 ± 8.2
Clean Air	30	9.2 ± 4.4	14.9 ± 5.2	27.8 ± 12.2	27.3 ± 10.6

[a] 31 week exposures

[b] 39 week exposures

[c] Percent of abnormally shaped sperm, average ± standard deviation.

Further studies are planned to a) identify, characterize, and quantitate the amount of genotoxic agents in diesel exhaust particles and their purified extracts, b) to evaluate the role of particle adsoprtion, metabolic transport, and activation, and c) to compare the genotoxic activities of purified agents in vitro and in vivo.

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TESTING FOR THE ABILITY OF MARINE DIESEL FUEL VAPORS TO INDUCE MICRONUCLEI OR SISTER CHROMATID EXCHANGES IN PERIPHERAL LYMPHOCYTES TAKEN FROM DOGS EXPOSED CONTINUOUSLY BY INHALATION FOR THIRTEEN WEEKS

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ABSTRACT

Purebred beagles were continuously exposed by inhalation to 0.05 mg/l or 0.30 mg/l marine diesel fuel (DFM) for thirteen weeks. Peripheral blood samples were taken during this exposure and after being stimulated to grow in vitro, the lymphocytes were examined for the presence of micronuclei, an indication of chromosome breakage, or sister chromatid exchanges (SCEs), an indication of chromosome rearrangement. No micronuclei above control levels were found in the exposed animals after one, nine or thirteen weeks exposure and no SCEs above control level were found after thirteen weeks of exposure to DFM.

INTRODUCTION

Marine diesel fuel (DFM) is used by a large number of ships operated by the United States Navy. It is prepared from natural crude oil and is composed of a mixture of a large number of different branched and cyclic hydrocarbons, including a small amount of benzene. To simulate the experience of ship crews who might be exposed to DFM in confined spaces for the duration of cruises, animals were exposed in inhalation chambers to DFM continuously for thirteen weeks.

Two groups each of six purebred dogs (three of each sex), 150 Fischer 344 rats (75 of each sex) and 140 C57Bl/6 mice (all female) were exposed continuously to 0.05 mg/l and 0.30 mg/l DFM, respectively, for thirteen weeks in Thomas Dome inhalation chambers. Another identical group of animals, housed separately, served as controls. During and at the end of the exposure period, various parameters were studied to determine the effect of DFM on the exposed animals. These parameters included hourly observations of the animals for signs of stress, counts of animal deaths, periodic animal weighings, periodic hematological and blood chemistry studies including red blood cell osmotic fragility measurements, and organ weighings and gross and histopathologic examinations of tissues of animals who died or were sacrificed immediately after the end of the exposure period, nineteen months postexposure or after the end of the animals' natural lifespan. The particulars of these observations are recorded in MacEwen and Vernot (1,2).

We report here the results of two other tests that were done. These were included to determine if DFM could induce cytogenetic damage to the dogs exposed by inhalation. Dogs

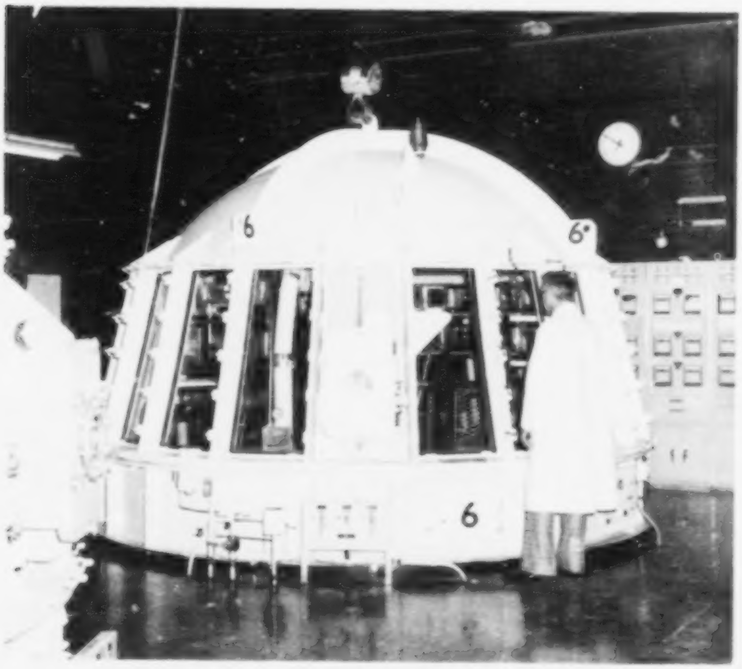

Figure 1. Thomas Dome Inhalation Chamber.

were chosen for the testing because we wished to develop general cytogenetic toxicologic testing methods that could be applied directly to humans. Dogs are large mammals as are humans and it is very easy to obtain a blood sample from a dog without otherwise harming the animal and so periodic samples can be taken from the same animals during a chronic exposure.

Periodic blood samples were taken from the dogs and lymphocytes stimulated to divide in vitro were examined for the presence of micronuclei, a very quickly and easily detected indication of induced chromosome breakage, and sister chromatid exchanges, an indication of induced chromosome rearrangement and the most sensitive test for mammalian cytogenetic damage known.

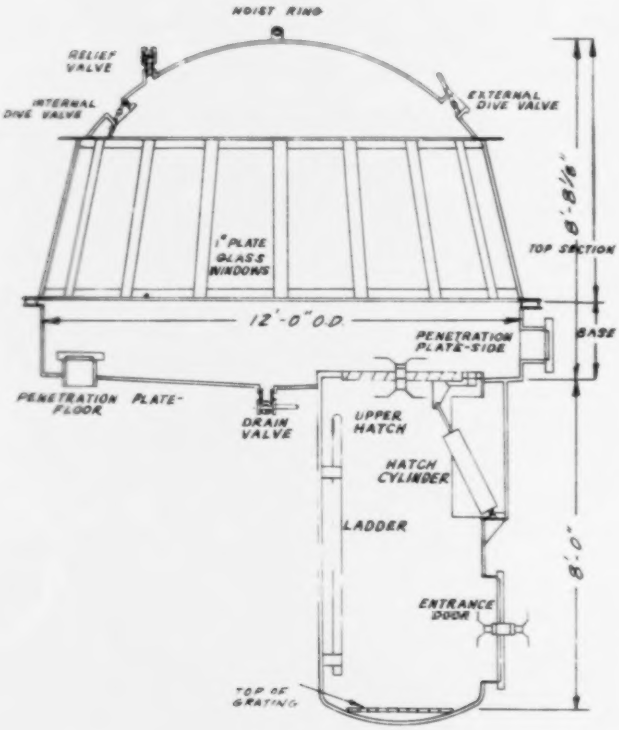

Figure 2. Cross Sectional View of a Thomas Dome and Access Air Lock

MATERIALS AND METHODS

Details of the methods of generation and monitoring of DFM vapors can be found in MacEwen and Vernot (1). One of the Thomas Dome inhalation chambers used for this study is shown in Figure 1 with a cross sectional diagram of it shown in Figure 2. There are eight of these domes at the Toxic Hazards Research Unit. This research was sponsored by the Aerospace Medical Research Laboratory, Aerospace Medical Division, Air Force Systems Command, Wright-Patterson Air Force Base, Ohio under Contract No. F33615-76-C-5005 with the University of California, Irvine. The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

To detect induced chromosome breakage in this study, the micronucleus test was used (3-7). Blood samples were taken from all the dogs involved in the study two weeks before exposure began and after one, nine and thirteen weeks of continuous exposure to DFM vapors. After each bleeding, the blood was immediately incubated for 75 hours (four cell doubling times) in McCoy's 5A cell culture medium at 37° C with a 5% CO_2 atmosphere in the presence of phytohemaglutinin to stimulate lymphocyte growth. The cells were then fixed onto microscope slides and stained with Giemsa blood stain. At least 500 cell nuclei were scored for the presence of accompanying micronuclei on each of two slides for each animal. Figure 3 shows a canine peripheral lymphocyte nucleus with accompanying micronucleus.

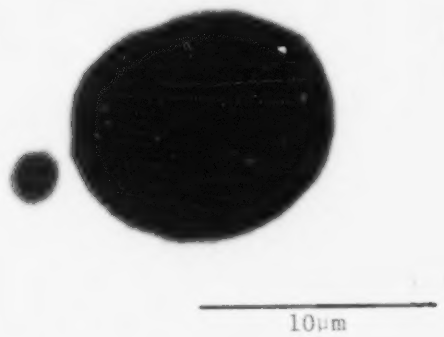

Figure 3. Canine Peripheral Lymphocyte Nucleus and Associated Micronucleus

To detect induced chromosome rearrangement, the sister chromatid exchange (SCE) test was used (8-11). Blood samples were taken from dogs after thirteen weeks of continuous exposure to DFM and incubated as described for the micronucleus test but for 49 hours and in the presence of bromodeoxyuridine. At that point colchicine was added and the cultures were incubated for four additional hours to trap the cells in metaphase. After this time the cells were swollen and fixed onto microscope slides and stained with Hoechst 33258 and Giemsa blood stains. Cells in metaphase were then examined for the presence of induced sister chromatid exchanges. Figure 4 shows a peripheral lymphocyte cell in metaphase that had been taken from an unexposed dog. The metaphase cell has been spread and stained to show the presence of sister chromatid exchanges.

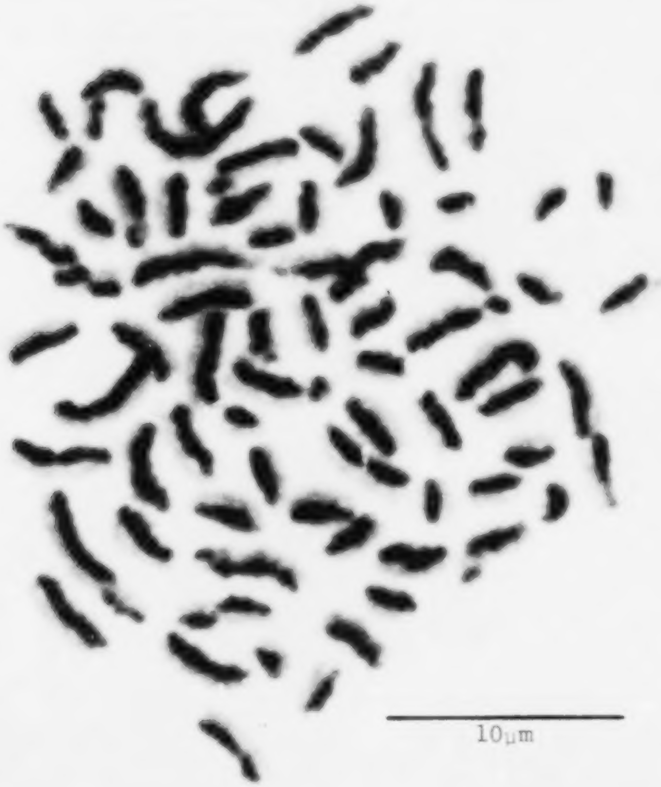

Figure 4. Canine Peripheral Lymphocyte Metaphase Chromosomes Stained to Reveal Sister Chromatid Exchanges.

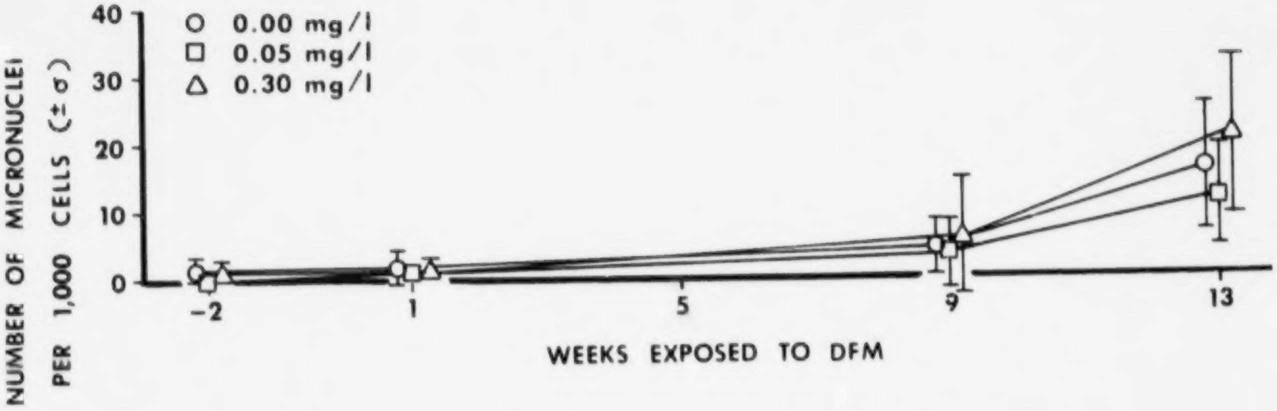

Figure 5. Induction of Micronuclei in Peripheral Lymphocytes of Dogs Exposed by Inhalation to Marine Diesel Fuel.

RESULTS

The results of our tests for the presence of induced micronuclei in the peripheral lymphocytes of the dogs exposed by inhalation to DFM are shown in Figure 5. Each point shown is the average count for all six animals exposed at each dose level. No differences were seen between males and females. No micronuclei above background counts were found to be induced by the dose levels of DFM vapors used in this study. All counts were unusually high at the thirteen week sampling. These slides contained an unusually high amount of cell debris which led to this result.

The results of our test for the presence of induced sister chromatid exchanges in lymphocytes of some of the male dogs involved in this study are shown in Table 1. Values are shown for five individual animals. No SCEs above background were found to be induced by the dose levels of DFM tested.

Table 1. Induction of Sister Chromatid Exchanges in Peripheral Lymphocytes of Dogs Exposed by Inhalation to Marine Diesel Fuel

Animal Identification Number	Exposure Level (mg/1)	Number of Metaphases Examined	Number of Exchanges/ Chromosome	Number of Exchanges/ Cell
X 48	0.00	56	0.0471	3.67
X 88	0.00	53	0.0573	4.47
X 52	0.05	12	0.0442	3.45
Y 10	0.05	2	0.0147	1.15
X 14	0.30	20	0.0600	4.68

DISCUSSION

The major purpose in doing this study was to establish procedures and techniques for performing cytogenetic tests with peripheral lymphocytes obtained from dogs. We wished to develop an animal model for future direct cytogenetic testing of humans (12,13) using dogs which are also large mammals. Stetka et al (14) have done similar work with rabbits. Some direct testing of humans using the sister chromatid exchange test has already been done (15-17), but we feel that carefully controlled animal exposure studies are needed to validate such testing. This study of the effect of DFM on dogs is a beginning in doing such a validation.

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Session VII

CARCINOGENIC EFFECTS OF EXPOSURE TO DIESEL EMISSIONS

Chairman:

Dr. Michael Pereira

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CARCINOGENICITY OF DIESEL EXHAUST PARTICLES BY INTRATRACHEAL INSTILLATION-DOSE RANGE STUDY[1]

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ABSTRACT

A study to determine the carcinogenicity of diesel exhaust particles, extracts of these particles, and other materials included for risk assessment purposes has been initiated. All materials under study will be administered to hamsters by intratracheal instillation. Prechronic dose range studies of diesel exhaust particles suspended in gelatine-saline and of particles bound to hematite (Fe_2O_3) have been carried out. Hamsters were treated for 15 weeks with test materials and killed 5 weeks later for histopathologic evaluation. Effects of treatment on weight gain, survival and induction of pathologic changes have been determined. The results will be used to select doses for lifetime carcinogenicity trials.

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[1] This study was initiated under EPA Grant R806326-01-1 and continued under Grant R806929-01-0. Dr. Donald E. Gardner, Inhalation Toxicology Branch, Health Effects Research Laboratory, EPA, Research Triangle Park, serves as Project Officer.

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The study we are reporting was carried out as part of EPA's program effort to assess the health effects of diesel exhaust emissions. Our overall program plans include the evaluation in hamsters of some of the matrix of materials of interest to EPA; namely, diesel exhaust emission extract, coke oven extract, roofing tar extract, and cigarette smoke condensate. However, our specific test system, intratracheal instillation in the hamster, is well adapted for studies of particulates and affords us the opportunity to also evaluate the carcinogenic properties of diesel exhaust emission particles as such.

This report describes results obtained in a dose range study with diesel exhaust particles (DP). Similar studies on the four extracts and condensates previously mentioned are in progress.

The diesel exhaust particles from a 5.7L Oldsmobile engine were provided to us by the Environmental Sciences Research Laboratory of EPA at Research Triangle Park. Their method of generation and collection and their chemical composition has been described. The preparation of our dosage forms, analysis of particle size distribution and quantification of dose levels was carried out at our institution and is described in a separate paper (See J. Graf, this volume).

Our bioassay protocol calls for the administration of test substances by intratracheal instillation in hamsters for 15 weeks, sacrifice of individual animals when they appear moribund, complete necropsies of each animal, and final diagnoses by histopathologic examination of tissue sections.

A preliminary dose-range study was carried out in order to provide an estimate of a maximum tolerated dose to be used for dose setting in the subsequent lifetime carcinogenicity assay. Since we had no good estimate of the inherent toxicity of the diesel exhaust particles in this model and anticipated the possibility of delayed or cummulative toxicity on the weekly dose schedule, we decided to carry out the dose range test utilizing the same 15-week dose period and to sacrifice surviving hamsters five weeks following the last weekly dose.

Initial test groups were large, consisting of 50 male hamsters per dose. Hamsters were received from Engle Animal Laboratories at 5 weeks of age and placed on test at 15 weeks of age. Animals were observed twice daily during the week and once daily during week-ends. They were housed three per cage in polycarbonate cages which were changed twice per week. Hamsters were fed Wayne Lab Blox and AbSorbDri was used as bedding material. All animals were weighed once per week during the period of the experiment.

The design of the dose-range study is shown in Figure 1. All groups were treated once weekly by a 0.2 ml intratracheal instillation containing the indicated quantity of test or control substance with the exception of the 10 mg/wk diesel particulate and diesel particulate plus ferric oxide groups which were treated twice weekly.

There were few signs of systemic toxicity in any of the diesel exhaust particle test groups. Deaths during the treatment and holding periods were generally 6% or less per group, and this is typical for this method of administration. There were no deaths in Groups 1-4 during the 5-week post treatment holding period. There were only 2 deaths in the diesel particulate-ferric oxide groups in the same period, 1 in Group 5 and 1 in Group 7. Mortality in the benzo(a)-pyrene positive controls was higher throughout, totaling 38% in the high dose B(a)P group (Group 9) and 12% in Group 10. Total mortality in the ferric oxide control and vehicle control groups was 6% and 10%, but no animals in either group died during the post treatment period. None of the untreated shelf control animals died.

Weight gains during the period of the experiment were similarly unaffected by treatment. All groups gained weight, though Group 1 and Group 9 animals gained slightly less than animals in other groups. High dose animals frequently lost weight during the first 2-3 weeks of treatment and then appeared to recover and gain weight.

Ten animals from each group were processed for complete histopathologic examination. There was little non-respiratory tract pathology observed. A liver lesion of moderate degree was found in five of the high dose B(a)P hamsters *(pericholangitis)* and this may be reflective of the other signs of toxicity found in this group.

Lungs and tracheas of from 10-25 hamsters per group have also been examined. A variety of lesions have been found which is probably not surprising in view of the large doses administered and of the apparently long retention time of even a single dose of intratracheally administered diesel exhaust particles. A gross indication of prolonged retention was obtained by administering single doses of 5 mg of diesel particles to a group of hamsters. Individual animals were killed at 1 hour, 8 days, 30 days, and 60 days

FIGURE 1

EXPERIMENTAL DESIGN

Group	Treatment	Concentration*	Frequency
1	Diesel Particles	5 MG/Instillation	2X/Week
2	Diesel Particles	5 MG/Instillation	1X/Week
3	Diesel Particles	3 MG/Instillation	1X/Week
4	Diesel Particles	1 MG/Instillation	1X/Week
5	D.P. + Fe_2O_3	5 MG/Instillation	2X/Week
6	D.P. + Fe_2O_3	5 MG/Instillation	1X/Week
7	D.P. + Fe_2O_3	3 MG/Instillation	1X/Week
8	D.P. + Fe_2O_3	1 MG/Instillation	1X/Week
9	B(a)P+ Fe_2O_3	3 MG/Instillation	1X/Week
10	B(a)P+ Fe_2O_3	1.5 MG/Instillation	1X/Week
11	Fe_2O_3 in Vehicle	5 MG/Instillation	1X/Week
12	Vehicle Control		1X/Week
13	Shelf Controls	NO INSTILLATIONS	. . . . . .

*Concentration refers to quantity of diesel particles, benzo(a)pyrene, or of Fe_2O_3 when only single materials were administered. When test materials were given with Fe_2O_3 the concentration is that of each component administered as 1:1 mixtures by weight.

post treatment. At 1 hour [Figure 2] the trachea and all lobes of the lung show the presence of particles and this can better be seen after trimming [Figure 3]. At 30 days there appears to be some clearance from the lungs [Figure 4] and a good deal from the trachea [Figure 5].

Histologic sections of the lungs of hamsters at these time points show that at one hour the particles are mostly localized with little material in alveolar spaces [Figure 6]. By 30 days [Figure 7] and at 60 days [Figure 8] there are particles in alveolar spaces and a chronic cellular response including hyperplasia in areas where particles are present.

In the dose response study where animals received either 15 or 30 intratracheal instillations, a variety of types of lung pathology was seen in some animals of all dose groups. A shelf control hamster [Figure 9] can be compared to a vehicle control hamster [Figure 10]. Treatment with vehicle produced multifocal areas of mild adenomatous hyperplasia but no inflammatory reaction. Ferric oxide in vehicle produced pneumonia in addition to adenomatous hyperplasia [Figure 11].

Benzo(a)pyrene and ferric oxide showed a more localized distribution [Figure 12] of the ferric oxide in bronchial regions as compared with diesel particles. A granulomatous lesion can also be seen. Tumors were found in some of the B(a)P-ferric oxide animals upon microscopic examination. There included a carcinoma *in situ* in a main bronchus [Figure 13], a sarcoma in the trachea [Figure 14] which extended around cartilage, and a squamous cell carcinoma of the trachea [Figure 15].

Two responses were present in the lungs of animals treated with diesel particles alone. First, adenomatous hyperplasia [Figure 16] was present in the region of terminal bronchioles and adjacent alveoli. This was more severe and extensive than in vehicle controls and was often associated with metaplasia to ciliated epithelium [Figure 17] and/or with squamous metaplasia [Figure 18], which was not present in the vehicle controls. Adenomas [Figure 19] were more numerous and larger than adenomas in vehicle controls.

The second lesion of DP-treated animals, a multifocal reactive response of alveolar walls, was not found in vehicle controls. This consisted of chronic pneumonitis [Figure 20], cellular hyperplasia [Figure 21], papillomatosis [Figure 22], macrophage infiltration [Figure 23], increased mitotic activity [Figure 24], and changes characteristic of atypia

[Figure 25] in individual cells. The significance of this lesion is still under discussion.

It should be kept in mind that this study was conducted for dose setting purposes preliminary to the conduct of a lifetime study. Conclusions as to the consequences that might be expected from long term holding of treated animals best awaits the conduct of the chronic study. However, the results of this dose-range experiment can be summarized as follows:

1. Diesel particles and diesel particles plus ferric oxide at the doses tested did not increase mortality above their respective control levels.

2. High doses of both preparations decreased weight gain in the first few weeks of treatment but had no appreciable effect on weight gain by the end of treatment.

3. There was no significant non-respiratory tract pathology in treated animals.

4. Lesions of the lungs were common in treated animals and included these findings:

    - Adenomatous hyperplasia is more severe and extensive in DP treated animals than in vehicle control animals.

    - Adenomas are more numerous and larger in the DP treated animals than in the vehicle control.

    - Metaplasia to ciliated epithelium and squamous metaplasia occurred in some DP treated animals but not in vehicle controls.

    - Severe multifocal reactive pneumonitis with hyperplasia and evidence of atypia occurred in DP treated animals but did not occur in vehicle controls.

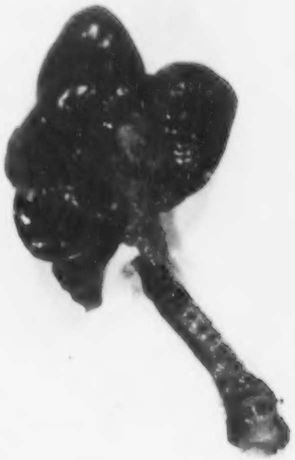

Figure 2. Intact Thoracic Viscera (1 Hour Post Instillation 5mg Diesel Particles.)

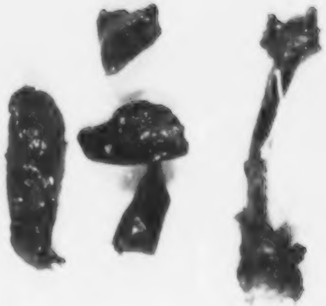

Figure 3. Dissected Trachea and Lungs (1 Hour Post Instillation 5 mg Diesel Particles.)

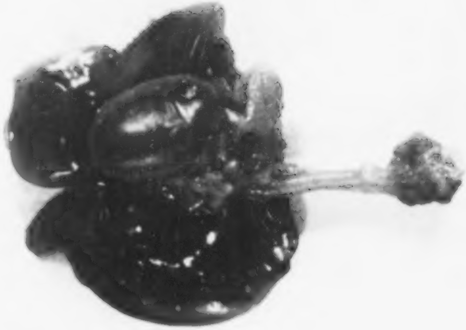

Figure 4. Intact Thoracic Viscera (30 Days Post Instillation 5mg Diesel Particles.)

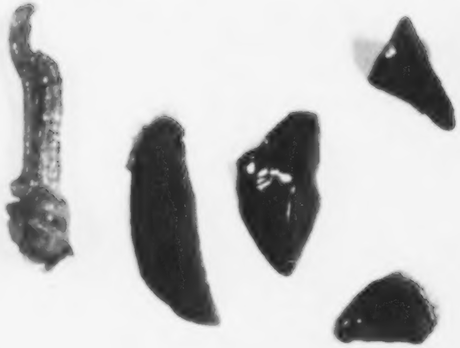

Figure 5. Dissected Trachea and Lungs (30 Days Post Instillation 5mg Diesel Particles.)

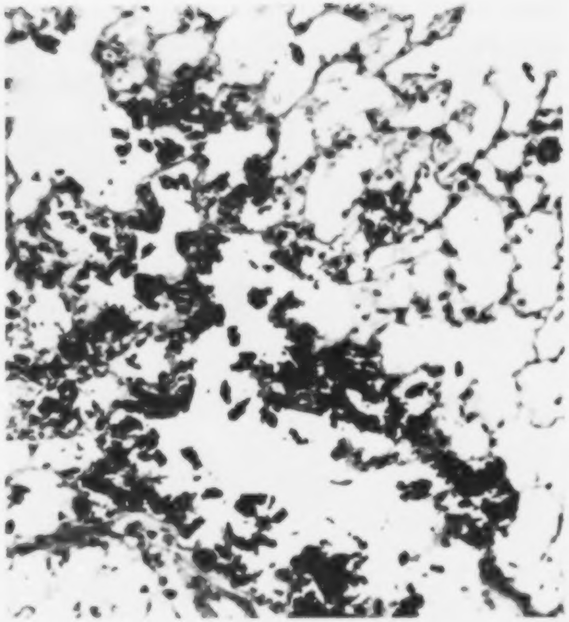

Figure 6. Section Through Lung of Hamster (1 Hour Post Instillation 5mg Diesel Particles.)

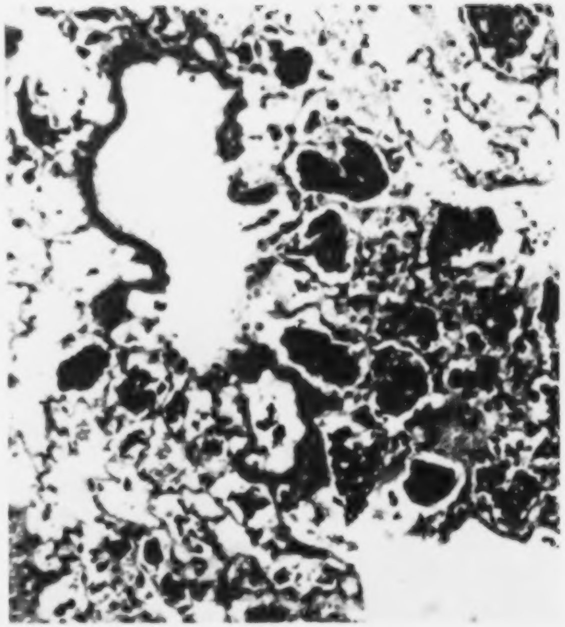

Figure 7. Section Through Lung of Hamster (30 Days Post Instillation 5 mg Diesel Particles.)

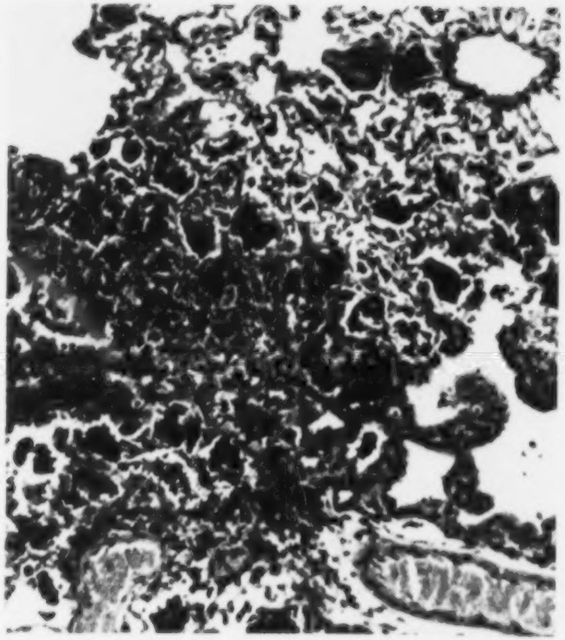

Figure 8. Section Through Lung of Hamster (60 Days Post Instillation 5mg Diesel Particles.)

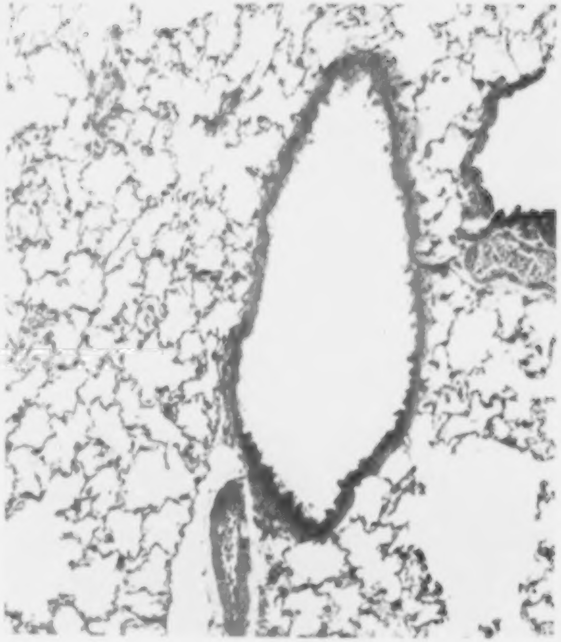

Figure 9. Shelf Control Hamster.

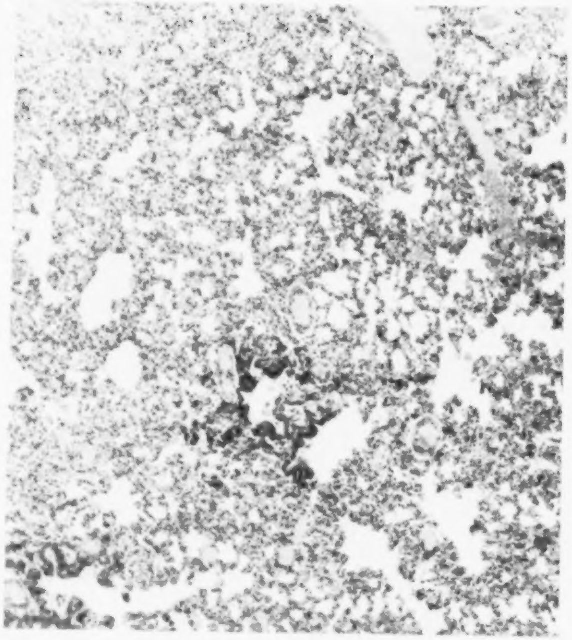

Figure 10. Vehicle Control Hamster.

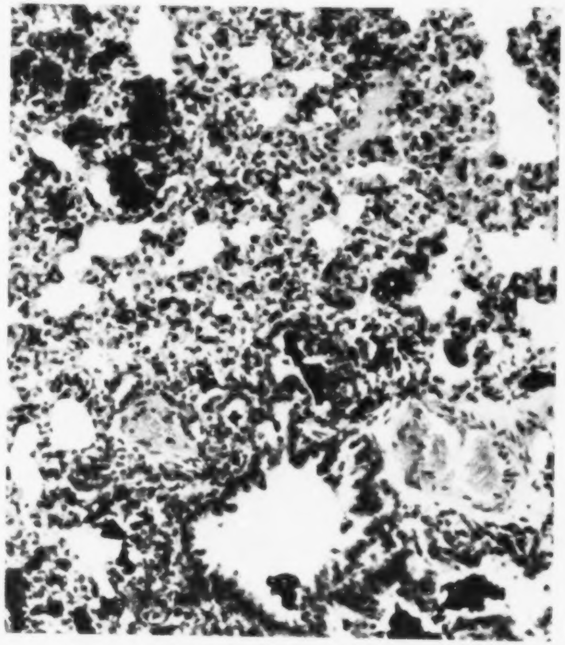

Figure 11. Lung Section from Hamster Treated With FE_2O_3 in Vehicle.

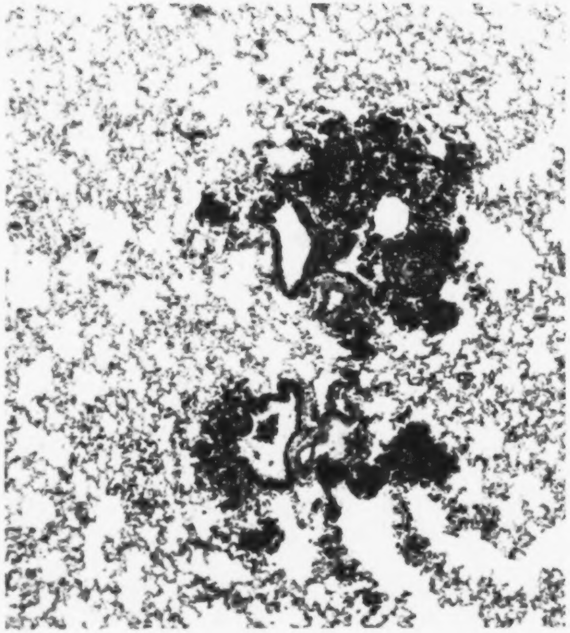

Figure 12. Benzo(a)Pyrene + FE_2O_3 Treated Hamster 3mg Each Per Week.)

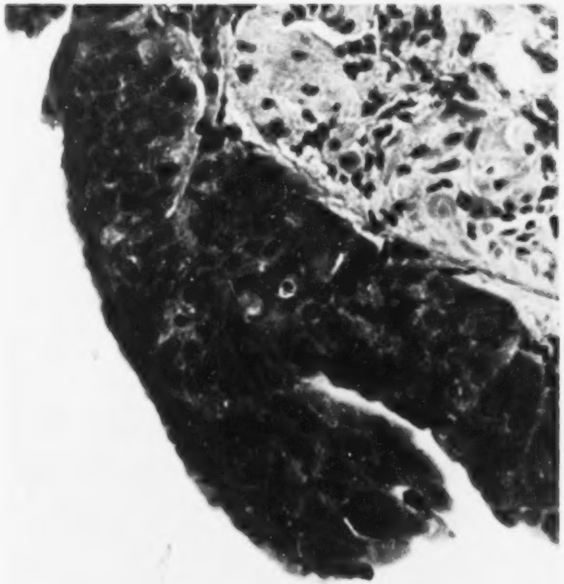

Figure 13. Carcinoma In Situ from Benzo(a)-Pyrene Treated Hamster.

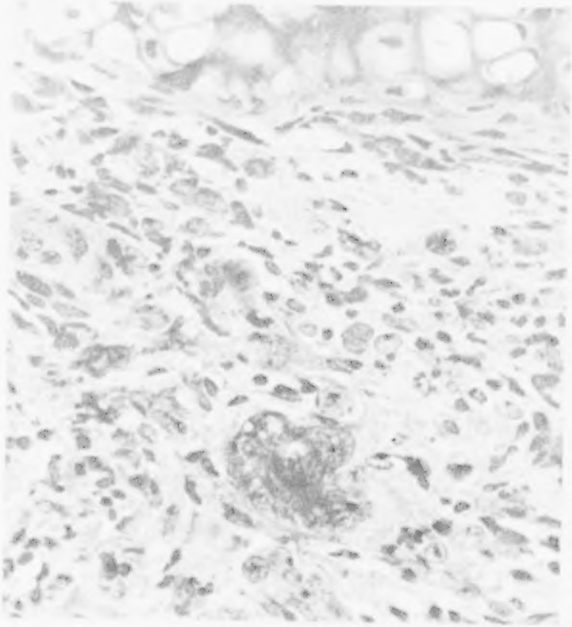

Figure 14. Sarcoma in the Trachea from Benzo(a)-Pyrene Treated Hamster.

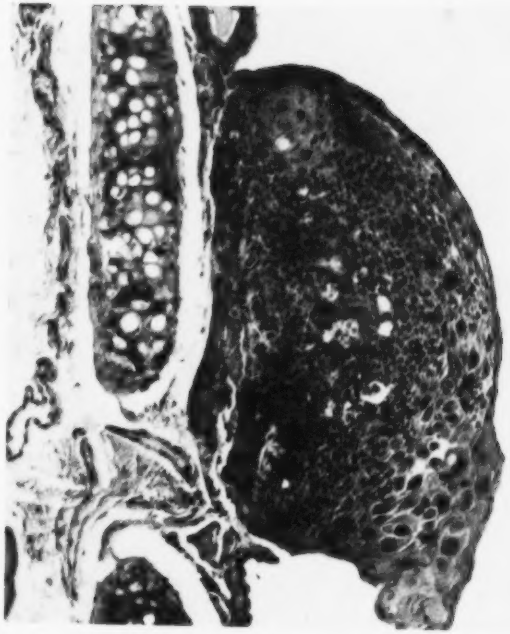

Figure 15. Squamous Cell Carcinoma in the Trachea of Benzo(a) Pyrene Treated Hamster.

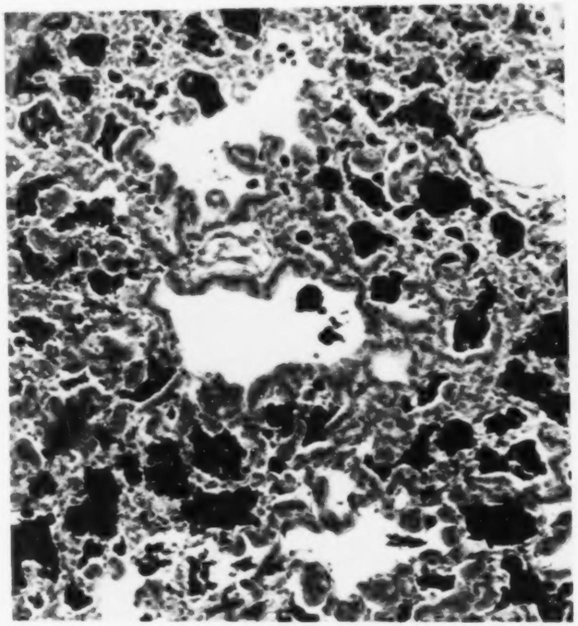

Figure 16. Adenomatous Hyperplasia from Diesel Treated Hamster (5mg/Week.)

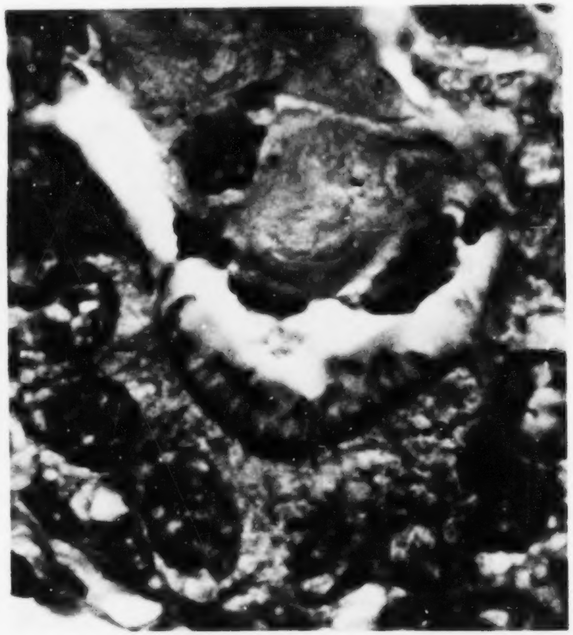

Figure 17. Metaplasia to Ciliated Epithelium from Diesel Particle Treated Hamster (5mg/Week.)

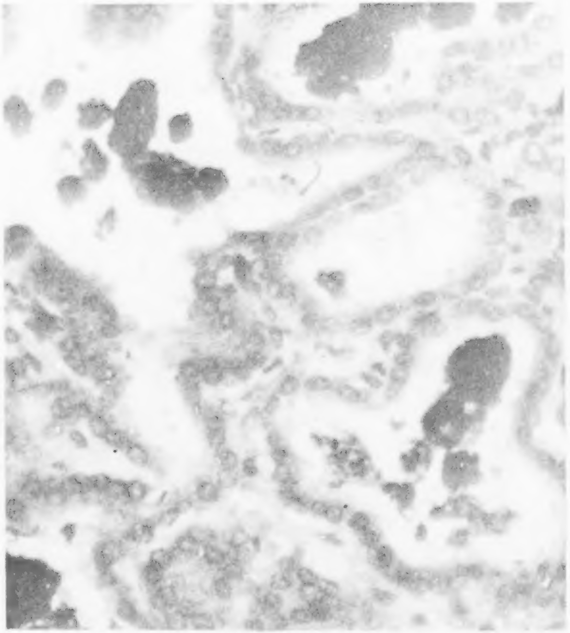

Figure 18. Squamous Metaplasia from Diesel Particle Treated Hamster (5mg/Week.)

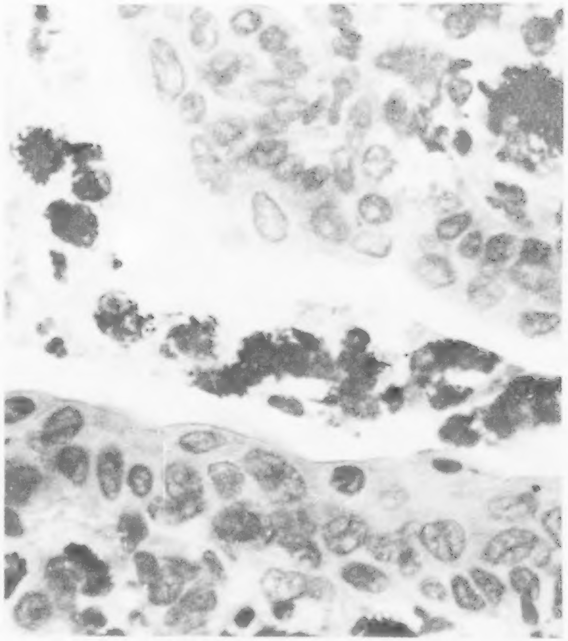

Figure 19. Pulmonary Adenoma from Diesel Particle (5mg/Week.)

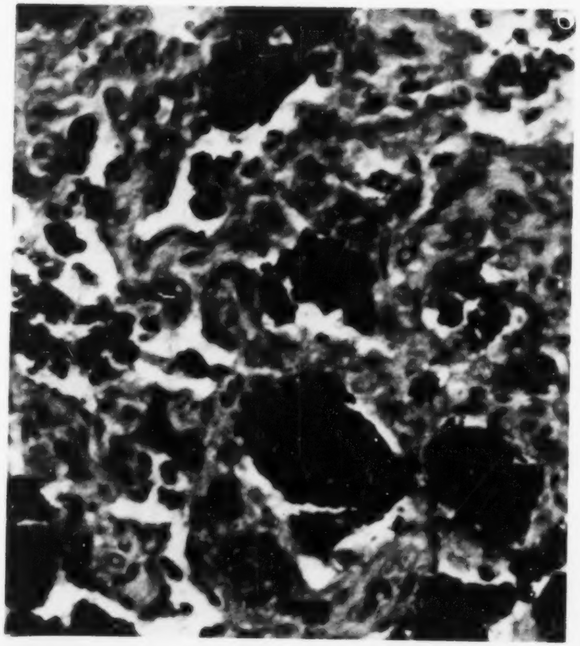

Figure 20. Chronic Pneumonitis from Diesel Particle Treated Hamster (5mg/Week.)

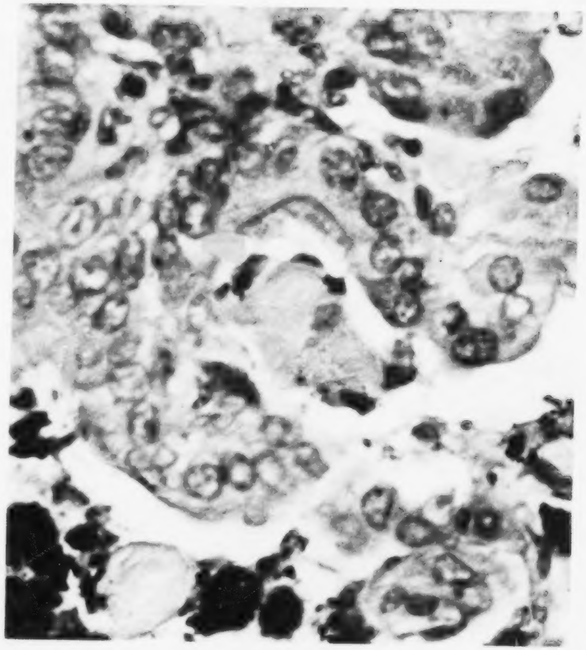

Figure 21. Cellular Hyperplasia from Diesel Particle Treated Hamster (5mg/Week.)

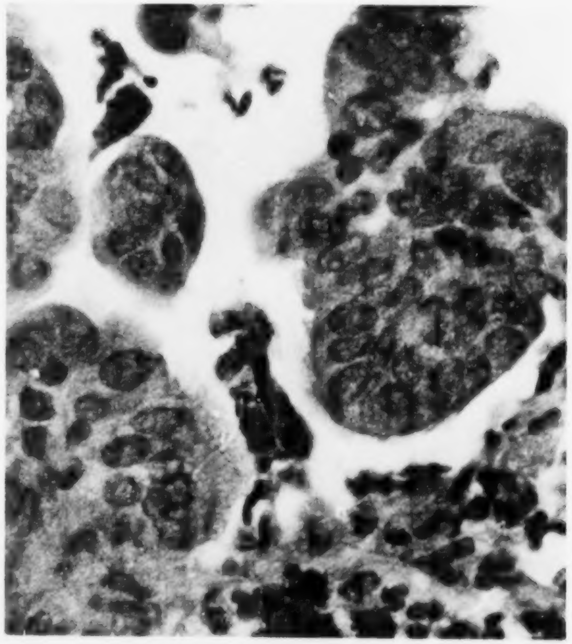

Figure 22. Papillomatosis from Diesel Particle Treated Hamster (5mg/Week.)

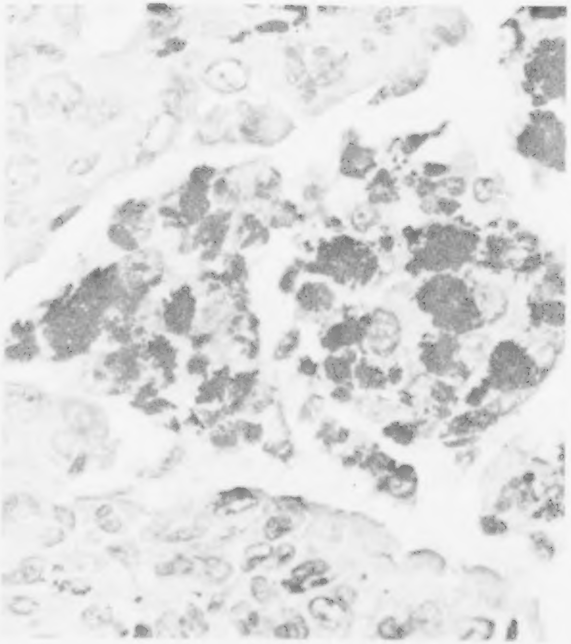

Figure 23. Macrophage Infiltration from Diesel Particle Treated Hamster (5mg/Week.)

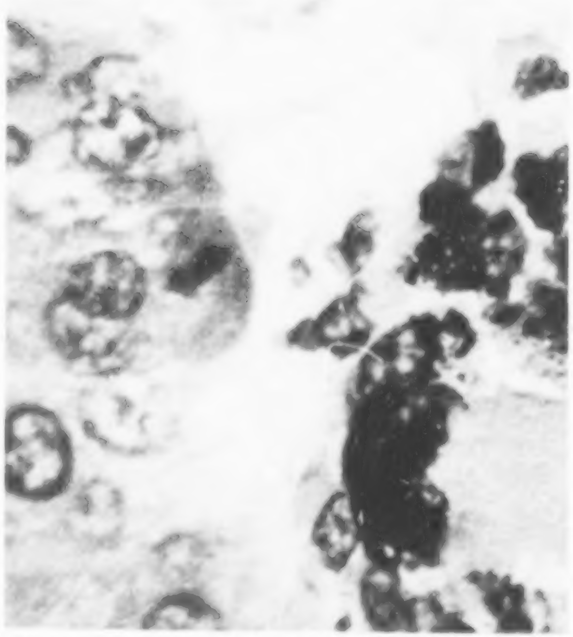

Figure 24. Mitotic Activity from Diesel Particle Treated Hamster (5mg/Week.)

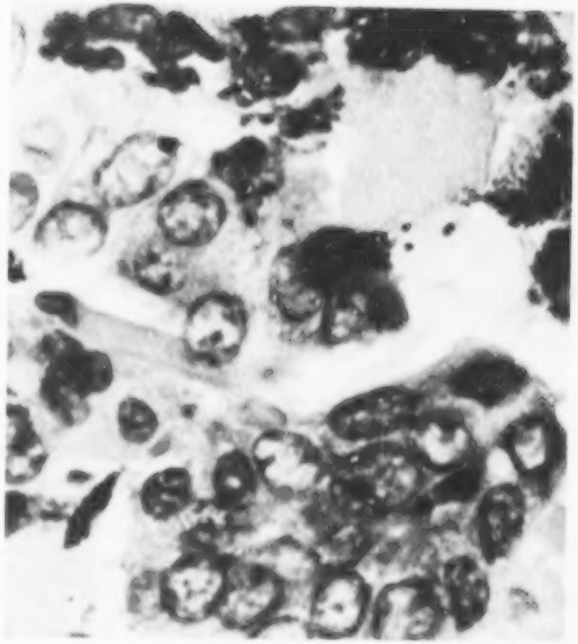

Figure 25. Cellular Atypia from Diesel Particle Treated Hamster (5mg/Week.)

THE TUMOR-PRODUCING EFFECTS OF AUTOMOBILE EXHAUST CONDENSATE AND OF DIESEL EXHAUST CONDENSATE

Mathematical-Statistical Evaluation of the Test Results[1]

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INTRODUCTION

This paper deals with the mathematical-statistical evaluation of animal experiments devoted to the following questions:

- To what extent is the carcinogenic or tumorigenic potency of automobile exhaust condensate (this means gasoline engine exhaust condensate) and diesel exhaust condensate dependent on the dose?
- How do the activities of these two condensates differ? In other words: What is the relative potency of these two substances?

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[1] With the support of the Bundesinnenministerium (Ministry of Internal Affairs) und des Umweltbundesamtes.

[2] In cooperation with H. Brune, Biologisches Laboratorium, c/o Vaselinwerk, Worthdamm 23-27, 2000 Hamburg 11, FRG, and G. Grimmer, Biochemisches Institut fur Umweltcarcinogene, Sieker Landstr. 19, 2070 Ahrensburg, FRG.

> What fractions (groups of substances) of the condensates are mainly responsible for the carcinogenic potency?
> To what extent can the potency of these mixtures be explained by fractions or single substances (as for example benzo(a)pyrene)?

In order to answer these and other questions, a working group[3] planned animal experiments (5), prepared AEC, DEC and subfractions of these condensates. In the first part of this note I shall discuss some fundamental mathematical-statistical aspects of analyzing these experiments, and then I give the results.

MATHEMATICAL-STATISTICAL METHODS OF THE EVALUATION

First of all one has to consider the dose-response-relationship of substances being tested. The response of a tumorgenic or carcinogenic substance can be measured by frequencies and by the tumor-induction period. So we have to consider two values, a qualitative one (= is there a tumor: yes - no) and a quantitative one (= time until a tumor is developed). These two values are not independent: Higher tumor-rates are correlated with lower tumor-induction times. In the following let us consider the parameter "tumor-rates".

One of the best tested carcinogenic substance is benzo(a)-pyrene (BaP). As an example for a typical dose-response curve look at Figure 1, which shows the relation between the 4 doses of BaP applied to 1200 mice and the tumor-frequencies (3). In 1860 G. T. Fechner (1) made use of a law which was first stated by E. H. Weber, in order to describe the relation between a stimulant and sensation. This law says that one gets a normal distribution function, if one transforms the doses into logarithms. So one gets the typical sigmoid dose-response curve, being symmetric about the 50%-point, as shown in Figure 2. This curve change over to a straight line if one transforms the frequencies P into probits Y with the help of the well known formula

$$P = \frac{1}{\sqrt{2\pi}} \cdot \int_{-\infty}^{Y-5} e^{-\frac{1}{2}u^2} \, du .$$

[See Figure 3 and (2)].

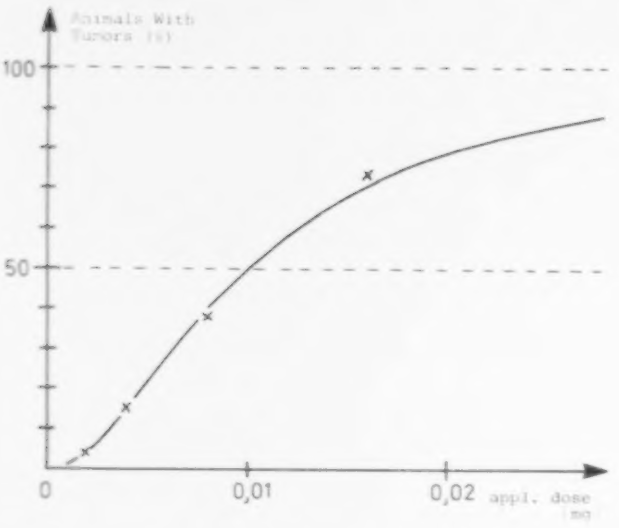

Figure 1. Tumor-Induction in Mice With Benzo(a)pyrene (3).

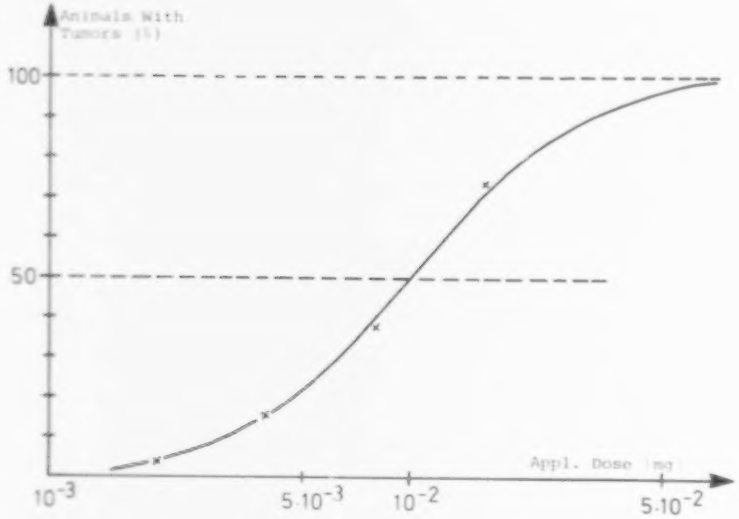

Figure 2. Sigmoid Dose-Response Curve for the Tumor Induction in Mice With Benzo(a)pyrene (3).

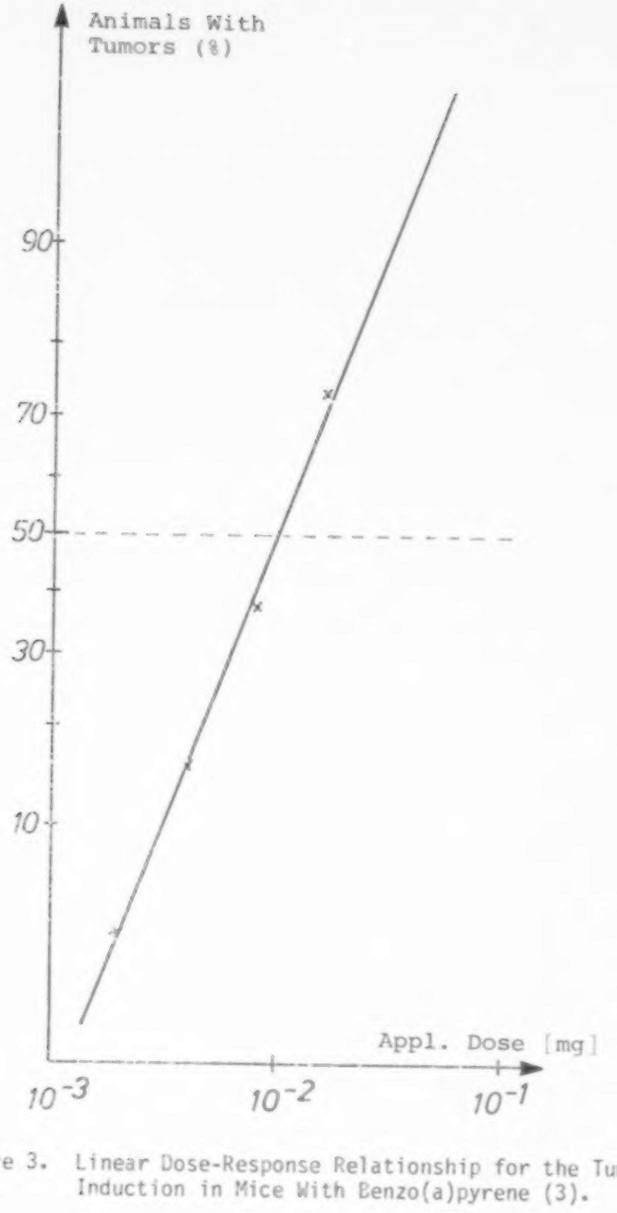

Figure 3. Linear Dose-Response Relationship for the Tumor-Induction in Mice With Benzo(a)pyrene (3).

Next we examine the possibility of giving a numerical comparison of the arcinogenic potency of different agents and of deducing the proportion of the potency of a mixture, which is deducible from the potency of its components. Assuming that the dose-response lines for carcinogens A and B are parallel in the transformed plane, we have a constant quotient d_B/d_A, where d_A = dose of carcinogen A and d_B = dose of carcinogen B, which result in equal number of animals with tumors. The following definition establishes a measure of relative carcinogenic potency:

Definition: Let dose d_A of substance A and d_B of substance B cause the same response. Then

$$\rho_{A/B} := d_B/d_A$$

is the relative potency of A with respect to B. We say that A is $\rho_{A/B}$- times as potent as B.

Figure 4 gives an example of substances A and B, where B is 10-times as potent as A.

Our next problem is to predict the potency of a mixture on the basis of potencies and amounts of its components. There are various theoretical possibilities (4), the simple case being that of similar action. In this case, the relative potency ρ of the mixture (with respect to any chosen standard) may be computed by

$$\rho = \sum_{\nu=1}^{n} \pi_\nu \rho_\nu$$

from the amounts π_ν and relative potencies ρ_ν (with respect to the same standard of the components $\nu = 1, 2, 3, \ldots, n$ (the amounts π_ν are taken as fractions of 1, i.e.,

$$\sum_{\nu=1}^{n} \pi_\nu = 1).$$

It is then possible to compute quantitatively the proportion of the potency of the mixture that is deducible from that of a component acting in a similar way.

Definition. Let A be a component of mixture M, the weight percentage of A in M be π, the relative potency of A (with respect to a standard) be ρ_A and the relative potency of M (with respect to the standard) be ρ_M. Then,

$$R = \pi \cdot \rho_A/\rho_M$$

is called the percentage of the potency of M deducible from A (acting in a similar way).

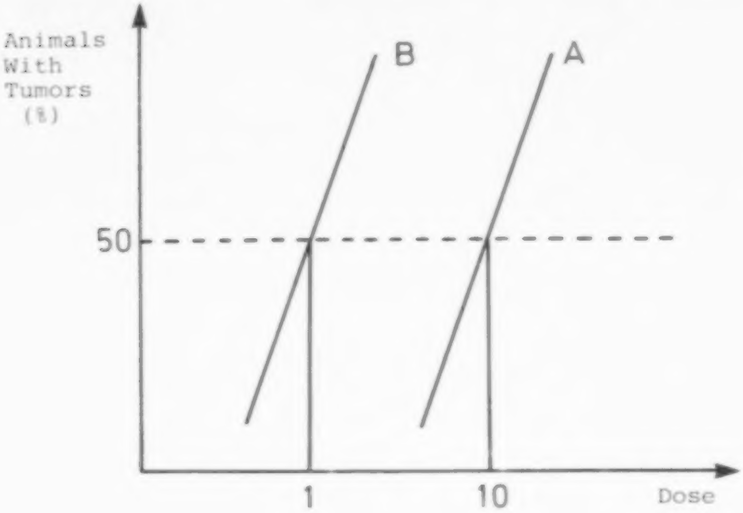

Figure 4. Definition of Relative Potency.
B is 10 times as active as A.

In our last example let us assume that A is a mixture with component B and that the weight-percentage of B in A is 5%. Application of the definition results in:

R = 10•5 = 50% of the activity of A is deducible from B.

The results of the evaluation, which are based on the proportions of tumor-bearing animals, may be misleading, since differences in mortality may occur as a result of, e.g., different toxicities of substances or of doses. Animals in experimental groups that are treated with more toxic substances tend to die earlier and are thus at minor risk of developing tumors. This fact may cause an underestimation of the tumorgenic potency of higher doses and of substances with higher toxicity.

In order to avoid such bias the analysis should be based on age-specific death and tumor rates. By considering these competing risks, the analysis should be able to separate the tumorgenic process from death due to other causes (5,7).

RESULTS OF THE EXPERIMENT

In order to answer the above questions 995 female CFLP-mice were dropped twice a week on the skin with AEC, DEC, BaP and a mixture of 15 carcinogenic PAH's, the weight proportion of which was chosen as in the AEC. The design of the experiment is contained in Table 1. We cannot discuss the methods of planning this design, especially hypotheses leading to the applied doses [see (4) and (5)]. The results of the experiment are shown in Table 2. Figure 5 shows that there are differences in death rates between the groups. Therefore the raw tumor-rates were transformed into age-standardized rates. These new rates are shown in Table 3. The test results are summarized in Figure 6. Differences in parellelism are not significant, therefore relative potencies could be calculated (see Table 4).

It is very interesting, that AEC is 42-times as active as DEC. (This means: In order to get the same tumor-frequencies one has to take a 42-times higher dose from DEC than from AEC.) Table 5 shows, what proportions of the condensates AEC and DEC can be explained by BaP and the 15 selected PAH's.

We may summarize: The mathematical-statistical evaluation shows:

1. The tumor-producing effect of AEC is 42-times as high as that of DEC.

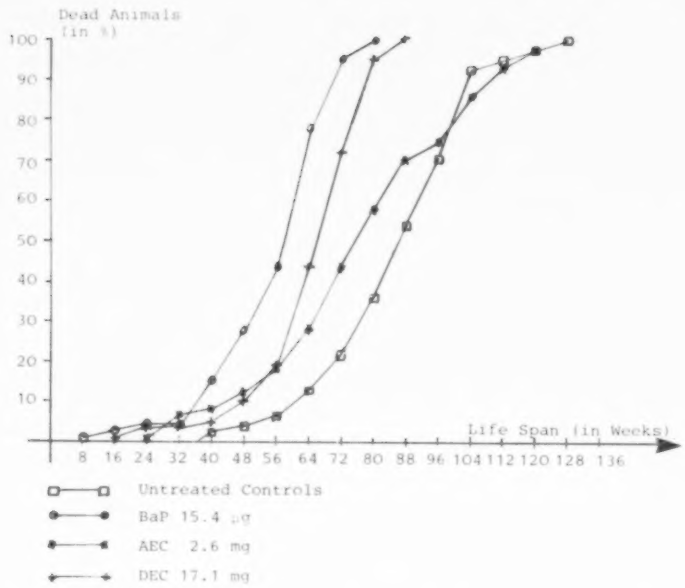

Figure 5. Development of the Death Rate for Some Test Groups.

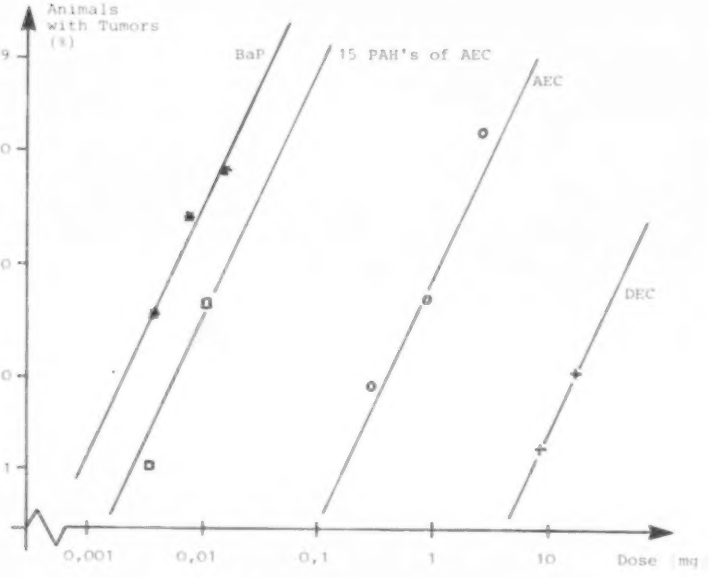

Figure 6. Age-Standardized Test Results.

TABLE 1. EXPERIMENTAL DESIGN TESTING THE TUMOR-PROMOTING ACTIVITY OF AEC, DEC AND SUBFRACTIONS

Test Group No./Substance	Applied Individual Dose in mg	Animals in Test Group
00 Control Without Treatment	-	80
01 Control Solvent	-	80
02 BaP	$3.85 \cdot 10^{-3}$	65
03 BaP	$7.69 \cdot 10^{-3}$	65
04 BaP	$15.38 \cdot 10^{-3}$	65
11 AEC	0.29	80
12 AEC	0.88	80
13 AEC	2.63	80
21 DEC	4.30	80
22 DEC	8.60	80
24 DEC	17.15	80
31 15 PAH's of AEC*	$3.5 \cdot 10^{-3}$	80
32 15 PAH's of AEC	$10.5 \cdot 10^{-3}$	80

AEC: Autombile Exhaust Condensate (Gasoline engine exhaust condensate)

DEC: Diesel Exhaust Condensate

*15 PAH's of AEC (weight proportion as in AEC): Benzo(c)-phenanthren (0.08 μg), Cyclopentenopyren (1.85 μg), Benzo-(a)anthracen (0.09 μg), Chrysen (0.21 μg), Benzo(b)fluoranthen (0.17 μg), Benzo(k)fluoranthen (0.06 μg), Benzo(j)-fluoranthen (0.09 μg), Benzo(a)pyren (0.30 μg), 1,12-Methylen-benzo(e)pyren (0.14 μg), 10,11-Methylenbenzo(a)-pyren (0.05 μg), Dibenz(a,j)anthracen (0.10 μg), Indeno-(1,2,3-cd)pyren (0.21 μg), Dibenz(a,h)anthracen (0.02 μg), M 300A (0.07 μg), M 300B (0.06 μg).

TABLE 2. RAW TEST RESULTS

Test Group No./Substance	Applied Individual Dose in mg	Evaluable Animals in Test Group	Average Length of Survival During Test (Weeks)	Animals With Tumors Absolute	%	Tumor Induction Period (Weeks)
00 Control Without Treatment	-	78	85	0	0.0	0
01 Control Solvent	-	78	76	0	0.0	0
02 BaP	$3.85 \cdot 10^{-3}$	64	78	21	32.8	74
03 BaP	$7.69 \cdot 10^{-3}$	64	68	39	60.9	61
04 BaP	$15.38 \cdot 10^{-3}$	64	55	57	89.1	44
11 AEC	0.29	78	80	8	10.3	72
12 AEC	0.88	79	78	35	44.3	72
13 AEC	2.63	78	64	65	83.3	52
21 DEC	4.30	75	80	0	0.0	0
22 DEC	8.60	76	81	2	2.6	102
24 DEC	17.15	71	77	9	12.7	76
31 15 PAH's of AEC	$3.5 \cdot 10^{-3}$	77	78	1	1.3	91
32 15 PAH's of AEC	$10.5 \cdot 10^{-3}$	75	76	29	38.7	73

TABLE 3. AGE-STANDARDIZED TEST RESULTS

Test Group No./Substance	Applied Individual Dose in mg	Evaluable Animals in Test Group	Animals With Tumors Raw Frequencies	Animals With Tumors Standardized Frequencies
00 Control Without Treatment	-	78	0.0	0.0
01 Control Solvent	-	78	0.0	0.0
02 BaP	$3.85 \cdot 10^{-3}$	64	32.8	28.4
03 BaP	$7.69 \cdot 10^{-3}$	64	60.9	70.2
04 BaP	$15.38 \cdot 10^{-3}$	64	89.1	85.8
11 AEC	0.29	78	10.3	8.5
12 AEC	0.88	79	44.3	34.8
13 AEC	2.63	78	83.3	93.5
21 DEC	4.30	75	0.0	0.0
22 DEC	8.60	76	2.6	1.9
23 DEC	17.15	71	12.7	11.5
31 15 PAH's of AEC	$3.5 \cdot 10^{-3}$	77	1.3	1.1
32 15 PAH's of AEC	$10.5 \cdot 10^{-3}$	75	38.7	33.0

TABLE 4. RELATIVE POTENCIES OF DEC, SUBFRACTIONS AND BAP RELATED TO AEC AND OF BAP RELATED TO DEC

Substance	Relative Potency	95% Confidence Limits
AEC	1	-
15 PAH's of AEC	68	46 - 101
BaP	187	127 - 286
DEC	0.024	0.015 - 0.035
DEC	1	-
BaP	7950	5210 - 13300

TABLE 5. PROPORTION OF THE POTENCY OF AEC AND DEC, WHICH CAN BE EXPLAINED BY SELECTED PAH'S.

Substance	Explained Proportion of the Potency	(%)	95% Confidence Limits
AEC	BaP	9.6	6.5 - 14.7
	15 PAH's of AEC	41.2	27.5 - 60.8
	Total-PAH-Fraction (6)	100	80.0 - 100
DEC	BaP	16.7	11.0 - 28.0

2. A synthetic mixture of 15 PAH's, known to be carcinogenic, shows 41% of the activity of the whole condensate AEC.

3. BaP can explain 9,6% of the activity of AEC and 16,7% of the activity of DEC.

Let me conclude with the remark, that further research is needed in order to show that these results are valid for other models, especially for inhalation studies.

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General Discussion

P. LEE: What is the biological basis for your frequency transformation? Is that a form of gamma function?

J. MISFELD: Yes, it is the interference of the gamma function.

P. LEE: What is the biological basis for this frequency transformation?

J. MISFELD: I don't know, the data used for this transformation. This calculation is valid when we have a single transformation in other experiments and not only in biological experiments.

SPEAKER: We have heard that both gasoline and diesel exhaust contain compounds which are not the conventional PAH's in that they do not require, for example, microsomal activation to express mutagenicity. Have you any data that the samples which you painted on the skin still contain such compounds or have they been lost in the extraction.

J. MISFELD: No, there may be other substances but we don't believe that there are other toxics in these extracts.

SPEAKER: Do you suggest that gasoline exhaust is more carcinogenic in this assay than diesel exhaust? Is this true also if you express it per mileage?

J. MISFELD: Yes.

J. HAFELE: You may have described the engines used, but I don't recall it. With respect to the gasoline engine was unleaded fuel used?

J. MISFELD: The engine was a Mercedes. The fuel in the gasoline engine was leaded.

J. HALEY: I understand that the latency period for tumors is a very strong function of the dose and then in the low dose ranges the latency period can exceed the life of the animal. Would you comment?

J. MISFELD: Yes, but if you take the two month selection period and conduct other inhalation exposures you get nearly the same results.

D. HOFFMANN: It appears that in mouse skin both the carcinogenic and tumorgenic response can be accounted in this study by the PAH or similar polar components. Based on your diesel data, do you think that what you are seeing in mouse skin is primarily due to the PAH's?

J. MISFELD: I an mot a biologist.

R. SCHRECK: Could you give us some description of the collection technique, particularly for the gasoline. Are you filter-collecting this, or is this strictly a condensate?

J. MISFELD: This was described earlier.

LONG TERM DIESEL EXHAUST INHALATION STUDIES WITH HAMSTERS

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ABSTRACT

Preceding a life-time inhalation exposure test, two range finding inhalation studies over three weeks and five months with diesel exhaust in varying concentrations were conducted to determine its acute and subacute toxicity. The animals (Syrian golden hamsters) received total exhaust and exhaust without particles 7-8 h/d and 5d/w. In order to evaluate the toxic effects, measurements and observations were made including some clinico-chemical and haematological parameters, body weight, organ weights and the histopathology of lung, trachea, liver and kidney. The evaluations indicated effects due to exposure, particularly evident in the lung. The range finding studies were carried out to determine an exhaust concentration which, in long-term exposures, would not lead to a significant shortening of the normal life expectancy of the hamsters.

Since then, a life-time experiment was started at an exhaust concentration of 1/7 (total exhaust and exhaust without particles) and has been going on for about 1o months. In addition to the exposure to diluted exhaust, some exposure groups receive in addition certain doses of a well-known carcinogen (diethylnitrosamine or dibenz(a,h)-anthracene), which, is expected to induce a tumor rate of 15 to 2o % in the respiratory tract. This animal model increases the probability of detecting slight carcinogenic or cocarcinogenic effects of inhaled diesel exhaust. Besides regular body weight checks, 22 different clinico-chemical parameters were measured after 8 months into the life-time experiment.

This paper deals with the first results of a small-scale inhalation study with Syrian golden hamsters on the biological effects of diesel exhaust emissions.

Our research project was initiated in 1977 as a pilot study at what is now the Fraunhofer Institute of Toxicology and Aerosol Research in Münster, West Germany. The study is part of a programm of a Task Group on the Airborne Carcinogenic Burden on Man, which was founded in 1970 and is now funded by the West German Federal Environmental Agency. To a small extent our particular research project was also supported by the Daimler-Benz Company.

The basic objective of our experimental study is to find an answer to the question as to whether and to what extent diesel exhaust is able to promote the development of lung cancer by way of inhalation. But other long-term biological effects are considered as well. Although the exhaust condensates of diesel and gasoline engines have already been proven to be carcinogenic in various non-inhalative animal models, no conclusive results are available on the inhalation hazard. Neither epidemiological studies nor animal inhalation exposure data exist up till now which would permit a definite statement on the carcinogenic potential of chronic inhalation exposures to man or animal.

Some results of in-vitro experiments using diesel exhaust condensate were published by EPA in late 1977 (EPA, Environmental News, Nov. 12, 1977). These data have given weight to the suspicion that the mutagenic effects seen in the Ames test are caused chiefly by the soot particles contained in the exhaust. Since then, our long-term inhalation, program having been designed at that time, focusses on the special role the soot particles of diesel exhaust may play in producing the effects under investigation.

Therefore, our pilot study employs diesel exhaust in two ways side by side. One line of experiments makes use of the original diesel exhaust diluted to suitable concentration levels, while the other line utilizes diesel exhaust void of any particulate matter.

The actual experimental exposures started in early 1978 and are made up of a successive series of three experiments. Each of these experiments determines the experimental conditions of the subsequent one. The first experiment was a preliminary investigation and lasted only 3 weeks. It simply served to provide a rough estimate of the acute toxicity of the total exhaust. Furthermore, it suited as a trial test run of the whole experimental set-up.

A variety of exhaust dilutions was used at this stage. Given as volume ratios of exhaust to clean air, mixtures of 1/2, 1/3, 1/5, 1/10, 1/15, and 1/20 were passed through the exposure chambers. The 1/2 dilution made it impossible to maintain representative temperature and humidity conditions in the chambers, so that this exhaust concentration was eliminated from subsequent investigations. In these preliminary exposures, only body weight checks and the general condition of the animals were taken as parameters to determine a toxic effect, and on that basis, no significant differences between exposed and control animals could be established.

In the subsequent investigation, which ran for over 5 months, an exhaust dilution was to be found that would give the highest concentration possible without impairing significantly the life expectancy of the animals during the third experiment which is currently under way as the final life-time study. Life span shortening could be caused by the effects of NO, NO_2, SO_2, CO and soot particles, all of which were administered in concentrations known to be toxic. The dilution ratios used here were 1/3, 1/5, and 1/10. The evaluation of the toxic effects from these exhaust dilutions was based on regular body weight checks, and on the results of some clinico-chemical and haematological analyses. Furthermore, certain organ weights were checked and some important organs were inspected by histopathological examinations.

In order to obtain a carcinogenic effect in inhalation experiments, it is necessary to exploit the relatively short life span of the rodent to the full. In view of the generally long induction period of carcinogens in humans, animal experiments must be conducted at high exposure concentrations just below the level of acute toxicity in order to compensate at least to some extent for the short life expectancy in comparison to man.

Since the concentration of known carcinogens in diesel exhaust is relatively low, only weak dilutions of the exhaust emissions may have a chance to produce experimental lung cancer by way of inhalation. For this very reason, an additional approach was designed for our inhalation experiments that may give us a better probability of finding at least a cocarcinogenic effect, if there is any.

It may well be that the effect of straightforward exhaust inhalation will remain within the flat range of the general sigmoidal dose-response relationship for tumor incidences. Even after exposure to the maximum permissible long-term concentration, the increase of the tumor incidence rate may still be insignificant for suitable sizes of animal test groups. However, by administering known carcinogens in addition, the basic tumor incidence rate may be shifted and moved into the steep slope of the dose-response relationship. By pre-inducing tumor rates of some 15 - 20 %, the additional exhaust exposure may then create significant changes in test groups of feasible size. As shown schematically on the graph (Fig.1), these changes need not be necessarily coergistic. One of us (F. Pott)

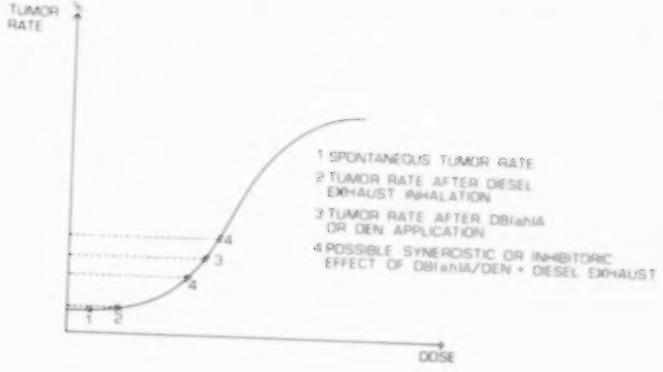
TUMOR RATE
%
1 SPONTANEOUS TUMOR RATE
2 TUMOR RATE AFTER DIESEL EXHAUST INHALATION
3 TUMOR RATE AFTER DB(a,h)A OR DEN APPLICATION
4 POSSIBLE SYNERGISTIC OR INHIBITORIC EFFECT OF DB(a,h)A/DEN + DIESEL EXHAUST
4
3
4
1
2
DOSE

Figure 1.

has shown in subcutaneous experiments with car exhaust condensate and benzo(a)pyrene that the exhaust condensate had a slight inhibitory effect on the known carcinogen.

Two different carcinogens are used to induce the 15-20 % tumor rate in our inhalation studies (Fig.15). Some additional groups receive the polycyclic aromatic hydrocarbon dibenz(a,h)anthracene. It is administered intratracheally once a week for 20 weeks. Other exposure groups receive a single subcutaneous injection of diethylnitrosamine (DENA). In contrast to the local effect of dibenz(a,h)anthracene, DENA has a systemic effect. In our particular case of Syrian golden hamsters, the effect is chiefly organ-specific and involves the respiratory tract. The application of DENA avoids minor lesions to the trachea, which cannot be completely ruled out in spite of the special intratracheal method used here.

The two carcinogens were actually administered to the exposed animals in the third experiment, the life-time exposure currently under way. In the 5-month experiment, half of the exposed animals received an intratracheal instillation of the noncarcinogenic PAH pyrene once a week for 20 weeks. The purpose of this procedure was to determine whether the application method as such causes a different reaction under diesel exhaust exposure in comparison with animals without instillations.

The 5-month experiment was completed by the end of 1978 and the subsequent life-time experiment has now been in operation for ca. 10 months. Throughout the project, Syrian golden hamsters are used as the test animals. This animal model is especially suited for the testing of the carcinogenic effects on the respiratory tract for the following reasons:

1. The spontaneous lung tumour rate is very low;
2. Infections in the respiratory tract are infrequent; and
3. The morphology of induced neoplasms seems to be very similar to tumors in the human respiratory tract.

A disadvantage of this animal model should not be ignored here: the incidence of kidney amyloidosis at the age of 1.5 to 2 years is rather common. This causes death in a percentage of the animals in this age group.

The exhaust used in the exposures is produced by a 2400 ccm Daimler Benz diesel engine. The engine, which is linked to an electrical dynanometer brake of the eddy current type, is mounted permanently on a spring-supported foundation. Its output and rotation are held constant at 16 kilowatts and 2400 r.p.m. The diesel fuel used is a European Reference Fuel and contains 0.36 % sulphur. The exhaust required for the exposures is drawn from a normal exhaust pipe into the mixing chambers. Part of it goes there directly, but another part passes first through a centrifuge, where the particulate matter is removed. Before the exhaust reaches the mixing chambers, its temperature is maintained above dew point, thus avoiding any condensation build-up and subsequent loss of certain substances in the exhaust. In the mixing chambers, the total exhaust and the exhaust without particles is diluted with cooled and dried clean air to the concentration required for the exposures. When the exhaust cools off in the mixing chambers, the dried diluting air takes care of the surplus moisture, so that even at this stage the dew point is not exceeded and water condensation is avoided. For safety reasons, the exhaust, which has passed through the centrifuge and has been diluted in the mixing box, will pass in addition through a fine filter. Thus, in case of an unforeseen breakdown of the centrifuge, the exhaust particles will then be removed by the filter, so that the exclusive exposure to the gas phase of the exhaust is maintained. The filter does not significantly influence the concentrations of CO, CO_2, SO_2, NO, NO_2 and total hydrocarbons. Measurements did not differ much from each other before and after the filter. Using supplementary mixing boxes, placed directly in front of the inhalation chambers the exhaust-air mixture can be further diluted to suit the exposure conditions.

A uniform horizontal flow is passed through the inhalation chambers. Special baffles and perforated diaphragms at the entrance and the exit openings guarantee an even distribution of the aerosol inside the chambers. In each chamber, there are 24 animals housed in wire cages on each of two levels. In each cage compartment there are three animals. The exhaust flow passes through the chamber at a rate of 80 litres per minute. Under this condition, the chamber atmosphere will be renewed about 20 times per hour. The chamber temperature is kept at 24-25 °C and the relative humidity at 50-60 %. The animals are exposed 7-8 h/d and 5 d/w. During the exposure period, there is no food in the cages; this is to keep the oral intake of diesel soot to a minimum.

As already mentioned, three exhaust dilutions were used in the 5-month inhalation study - the ratios were 1/3, 1/5, and 1/10 (Fig.2). One half of each dose group received the total exhaust, and the other half was exposed to the exhaust without particles. Of the 48 animals per dose group, 24 received an intratracheal instillation of 0.5 mg pyrene in 0.15 ml physiological saline solution. This mixture was administered once a week for 20 weeks. In order to obtain a suspension as homogeneous and finely distributed as possible, some Tween 20 - an emulsifier - was added to the saline solution. After adding the pyrene, the mixing was effected ultrasonically. The same method of emulsification is used in the current life-time exposures for making the dibenz(a,h)anthracene suspensions. An appropriate number of controls, some receiving instillations and some not, were kept in inhalation chambers ventilated by clean air.

The concentrations of the following gaseous com-

5 MONTH INHALATION STUDY WITH DIESEL EXHAUST

EXPERIMENTAL GROUPS	TOTAL EXHAUST			EXHAUST WITHOUT PARTICLES			CLEAN AIR
	1/3	1/5	1/10	1/3	1/5	1/10	
HAMSTER + PYRENE INSTILLATION (ITR)	24	24	24	24	24	24	24
HAMSTERS	24	24	24	24	24	24	24

Figure 2.

ponents in the exhaust were measured continuously in the inhalation chambers using a compact commercial measuring system: CO, CO2, NO, NO_2, NO_x, total hydrocarbons, methane, non-methane hydrocarbons and SO_2. In the following figures, the concentration of these gaseous components is shown separately for total exhaust and exhaust without particles. However, the differences between the two exposure atmospheres are very small. The concentrations in the highest and lowest dose group were as follows:

40-45 ppm and 11 ppm for CO
1.5 volume % and 0.5 volume % for CO_2 (Fig.3)
70 ppm and 20 ppm for NO
3 ppm and 0.5 ppm for NO_2 (Fig. 4)
13 ppm and 7 ppm for total hydrocarbons

Furthermore, about 5 ppm were measured in all inhalation chambers for methane. Since the methane portion in the exhaust itself is under 1 ppm, the methane concentration in the chambers is mainly generated by the animals (Fig. 5). The SO_2 concentration was ca. 13 ppm in the highest dose group and 2-3 ppm in the lowest (Fig.6). The gravimetrically determined particle mass concentration in the inhalation chambers was about 17 mg per m^3 for 1/3 dilution, approximately 11 mg per m^3

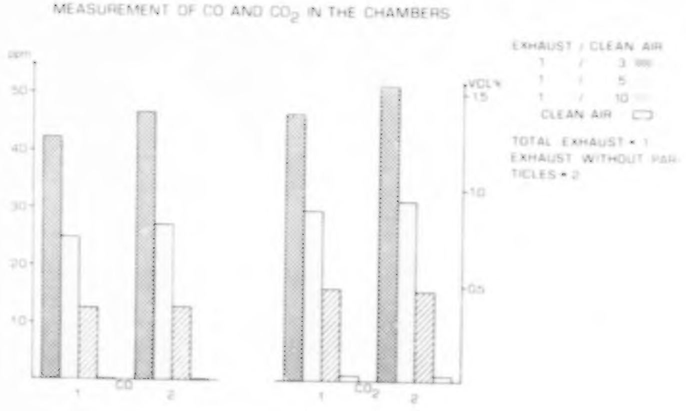

Figure 3.

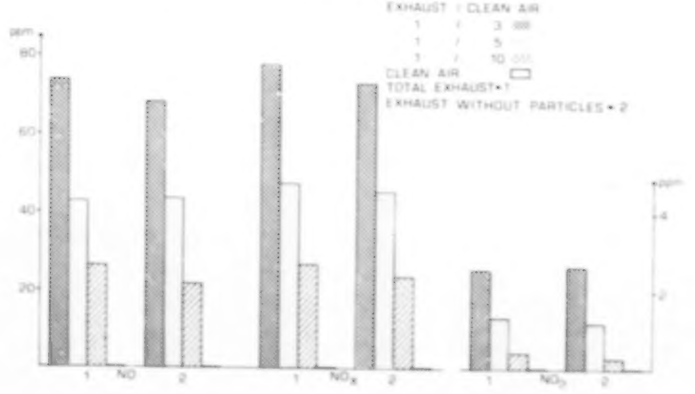

Figure 4.

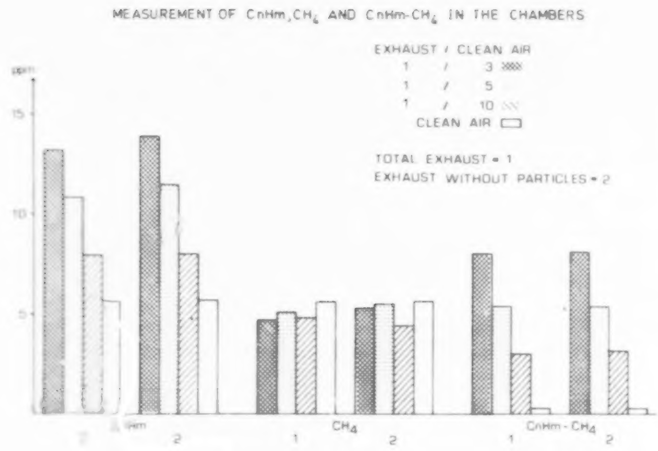

Figure 5.

for the 1/5 dilution and about 4 mg per m^3 for the 1/10 dilution (Fig.6). The particle size distribution of soot particles in exhaust was

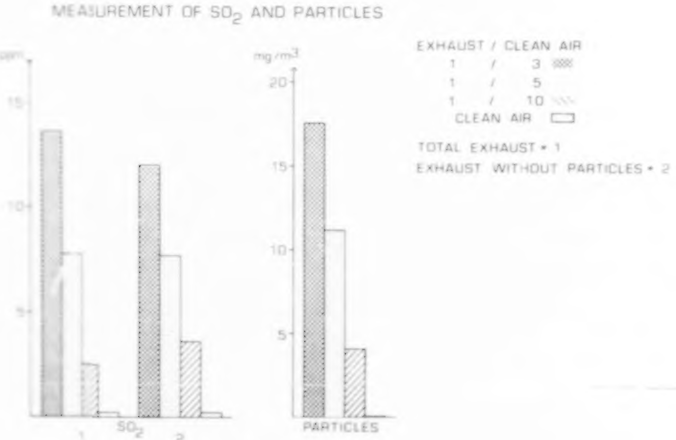

Figure 6.

determined just before the entrance to the inhalation chambers using an aerosol spiral duct centrifuge. The modal peak of the particle mass distribution had an aerodynamic diameter of 0.1 um (Fig.7).

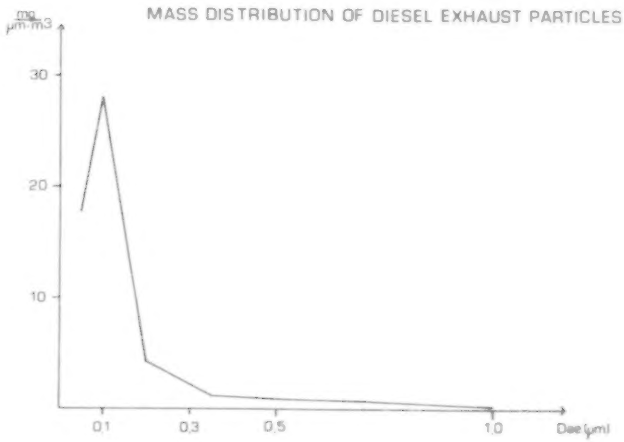

Figure 7.

After regular body weight checks every 2 weeks, 10 animals from each group were examined at the end of the 5-month study for the following parameters: erythrocyte and leucocyte counts, mean corpuscular volume of erythrocytes, total haemoglobin, CO-haemoglobin and methemoglobin, haematocrit, some enzymes in the plasma, such as two transaminases, GOT and GPT, alkaline phosphatase, serum urea, and finally, weights of lung, heart, liver, kidney and spleen. Lung and trachea as well as liver and kidney were also examined histopathologically.

In the following figures, the results of these examinations are separated into those groups which received intratracheal instillations and those groups which did not. The caption "itr" underneath the column diagram denotes those exposure groups which received instillations with pyrene; the number 1 represents those groups which were exposed to the total exhaust, and the number 2 represents those groups, which only inhaled the gaseous phase of the exhaust.

In comparison to the controls, the number of erythrocytes is somewhat decreased in all exposure groups (Fig.8). However, this difference is significant only for those groups, which received

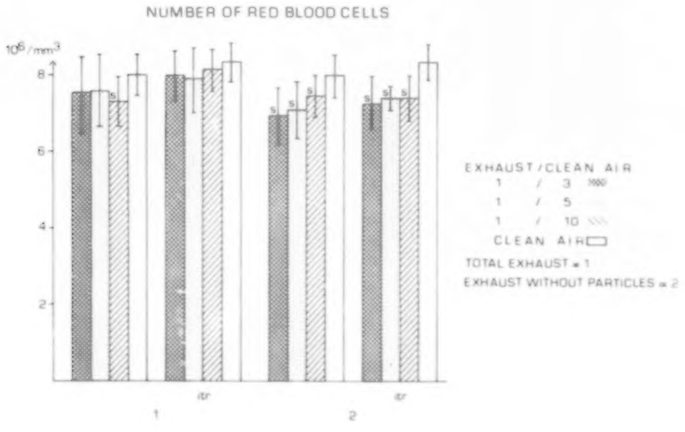

Figure 8.

the gaseous phase of the exhaust without the particles. In contrast, the mean corpuscular volume of the erythrocytes, when compared to the controls, is significantly higher for almost all exposed groups (Fig. 9). The haematocrit value for exposed animals, which should be low due to the small number of erythrocytes, shows this significantly only in the highest dose group (Fig. 10). The absence of this effect in the other dose groups is probably due to the fact that the reduced number of erythrocytes is compensated by the significantly higher mean corpuscular volumes. Since the haematocrit reproduces the cellular volume proportion of the blood, the influence the smaller number of cells should have, must have been neutralized by the larger volume of the cells. The haematocrit value of one exposure group could not be measured, because the measuring instrument had broken down and there was no blood

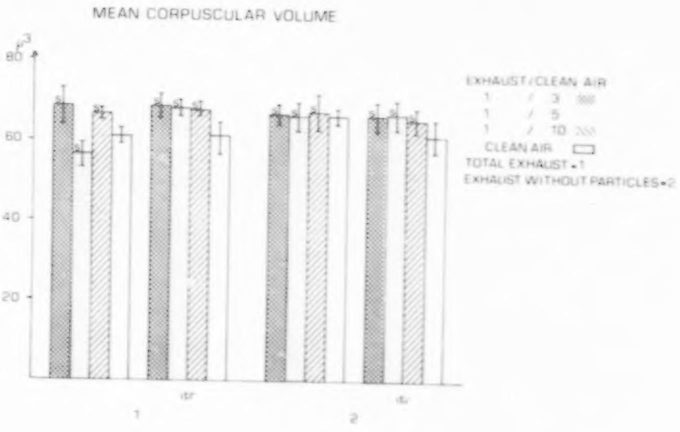

Figure 9.

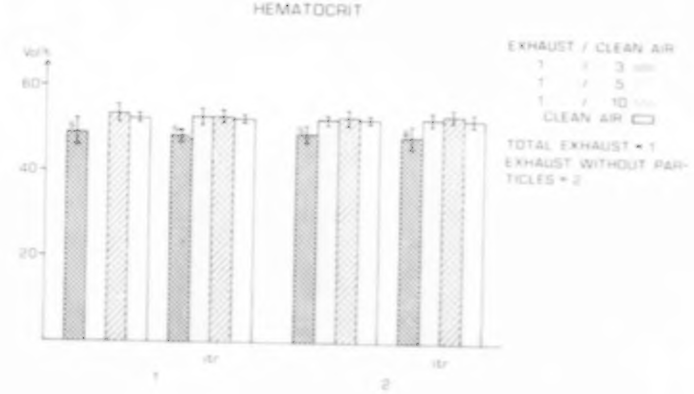

Figure 1o.

available for a repeat.

No significant difference between any exposure group and controls could be determined for the haemoglobin concentrations.

The methemoglobin and CO-haemoglobin values of the exposed animals appear to be higher than those of the controls; in the highest dose group they are considerably higher (Fig.11). However, all of these values still lie in a range where there is no serious risk to the transport of oxygen. The relatively large methemoglobin formation in each of the highest dose groups reflects the high NO-concentration in these exposures, as NO must be seen as an originating factor in the formation of methemoglobin. CO-haemoglobin could only be detected in the highest dose groups, and only then when the measurements were made immediately after the exposure. This is because CO-haemoglobin decomposes very rapidly.

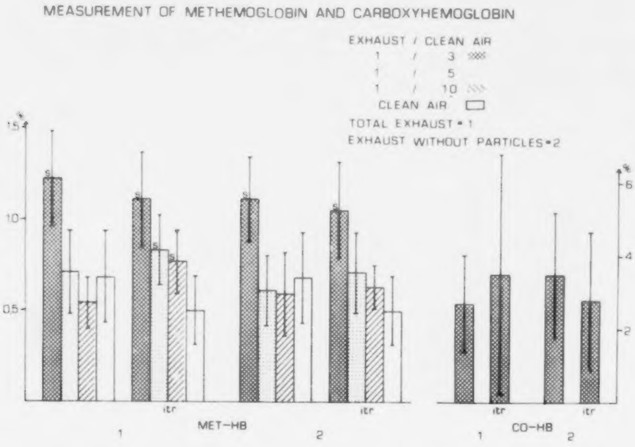

Figure 11.

In contrast, the increased methemoglobin values could be measured even after a weekend when no exposure took place.

The leucocyte count was apparently slightly lower in all exposure groups, in some cases even significantly, but no dose-dependent effect could be observed. Instead, the values for the different exposure groups varied randomly.

The results of the enzyme measurements, GOT, GPT, and alkaline phosphatase, show no clear indication of organ lesions. However, the blood urea content points to a kidney insufficiency. Animals, which were exposed to total exhaust, showed a significant increase in blood urea concentration (Fig.12). The histopathological examination of the kidneys could not, however, definitely confirm this insufficiency. Even the histological examination of the liver, the weights of heart, kidney, liver and spleen, and body weight checks indicated no difference to the controls.

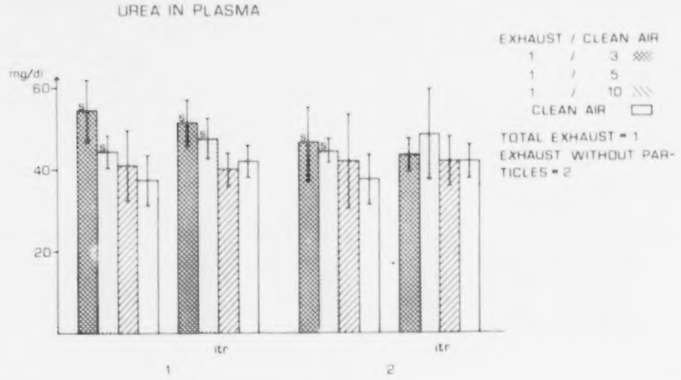

Figure 12.

An effect, which was clearly caused by the exposure to the diesel particles was found in the lung, the primary target organ of inhaled exhaust. A dose dependency could be observed even in a simple macroscopic examination of the lungs. They differed by their grey or black colouring, in relation to the increasing concentration of the soot particles. Even the lymph nodes in the lung, which were prepared for microscopic inspection, were clearly colored black. This findings indicate that the removal of the inhaled soot particles occurs to a noticeable extent by transport the lymphatic system. It appears possible that soot particles or various substances, which may desorb from the particles, may cause a burden on other organs by further lymphatic transport.

In the 5-month experiment, the wet and dry weight of the lungs was determined from 10 animals per exposure group. In all 3 dose groups, the lung wet weight expressed as a percentage of the body weight of animals exposed to total exhaust, was significantly increased in the two highest dose groups. This was also the case for those animals, which in addition received intratracheal instillations (Fig.13).

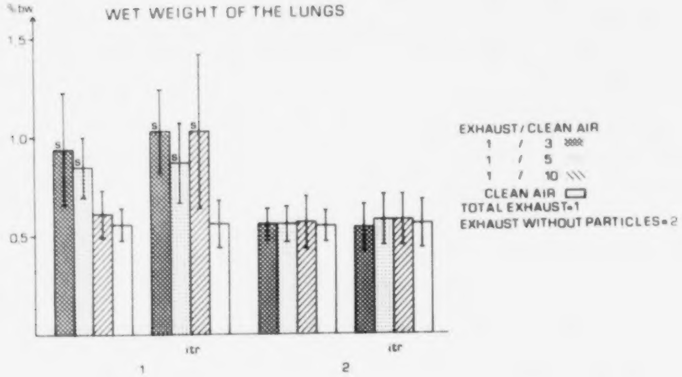

Figure 13.

On the other hand, those animals, which only inhaled the gaseous phase of the exhaust, had lung wet weights almost identical to the controls. Furthermore, no difference could be detected between the animals with and without instillations.

The same dose-dependent effect can be seen in the lung dry weight of those animals exposed to the total exhaust. Again, all animals, which inhaled exhaust without particles, showed no significant difference to the controls (Fig.14).

Since the increase in the wet weight and the dry weight of the lungs was of the same rate, and

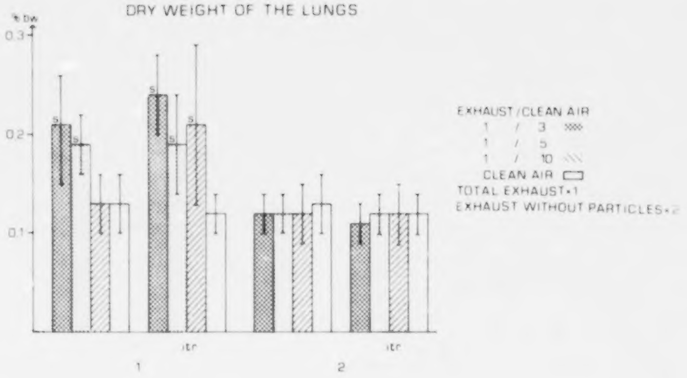

Figure 14.

since the water content of the lungs of these animals did not differ from that of the controls, it can be assumed that the increase in lung weight cannot significantly come from an oedema formation, but must be chiefly due to a tissue proliferation. This can be concluded, because the soot particles inhaled and deposited in the lung would account only to a small extent for the weight differences actually observed. Assuming a breathing volume of 100 ml per minute for the hamster and a particle concentration of 17 mg per m^3 in the highest dose group, then, after 5 months, the inhaled particle mass per animal would be about 70 mg without taking into account the very effective particle removal by the nose of the hamster. Assuming further a lung deposition rate of 50 %, which in reality is an excessive rate, then the particle mass deposited in the lung would only account for a maximum of 5-10 % of the increase of the absolute wet weight, and a maximum of 20-30 % in case of the dry weight.

In view of the histopathological examinations, the main cause of the increase in lung weight must be the inflamed, granulomatous, and proliferative alterations of the lungs of the exposed animals. However, only by means of a morphometric analysis of the lung tissue is it possible to make a dose-dependent quantification of the histopathological findings. Since this elaborate examination has not yet been carried out, only a general statement can be made at this time: the lesions of the lung tissue are much more pronounced in the highest dose groups than in the lowest. These lung alterations were not found in the animals, which inhaled only the gaseous phase of the exhaust, and, of course, not in the controls.

At the end of the 5-month experiment, a few animals from each group were saved. For another 9-10 months, they were kept under normal clean air conditions. After this period, the macroscopical and histological examination carried out on the animals which were exposed to total exhaust showed distinct particle depositions in the lung, including the animals exposed to the lowest dose. In particular, the histological picture of the lungs in the lowest dose group is partly marked by a strong proliferation of the terminal brochiolar epithelium into the alveolar spaces. It was not possible to detect this effect in the examinations directly after the exposure period of 5 months. It must be pointed out, though, that there were very few animals available for this late examination, and that it is not possible at this time to furnish proof of a causal relationship between the particle deposits and the adenomatous proliferation.

The results of this 5-month inhalation study constituted the basis for the design of the lifetime exposure test. In that investigation, there is a total of 18 exposure groups, with 48 animals each (Fig.15). The study is now into its tenth month. Six of these groups receive total exhaust, 6 other groups receive exhaust without particles. The remaining 6 groups serve as controls and receive clean air. The exhaust is given to all groups at the same dilution ratio of 1/7, exhaust in clean air. The details regarding the engine, inhalation chambers and exposure periods are identical to those employed in the 5-month experiment. After an exposure period of 2 months, two groups of each exposure condition, that is a total of 6 groups, received a single injection of diethylnitrosamine in two different doses. Another 6 groups received intratracheal instillations of a suspension of 2 different doses of dibenz(a,h)anthracene once a week for 20 weeks. Thus, tumor induction rates will be produced at two different dose levels. As

LIFETIME INHALATION STUDY WITH DIESEL EXHAUST

ITR. INST. / S.C.	CLEAN AIR	TOTAL EXHAUST	EXHAUST WITHOUT PARTICLES
WITHOUT	48	48	48
DB(a,h)A 6 MG (20 x 0.3 MG in 0.02 ML)	48	48	48
DB(a,h)A 2 MG (20 x 0.1 MG IN 0.15 ML)	48	48	48
PYRENE 2 MG (20 x 0.1 MG IN 0.15 ML)	48	48	48
DEN 1.5 MG/KG BW	48	48	48
DEN 4.5 MG/KG BW	48	48	48

DB(a,h)A = DIBENZ(a,h)ANTHRACEN DEN = DIETHYLNITROSAMINE

Figure 15.

mentioned earlier, with animals treated in this way, there is an increased probability of detecting a carcinogenic or cocarcinogenic effect of inhaled diesel exhaust with statistical significance. In order to exclude any effects caused simply by the method of intratracheal instillation, one group of each exposure condition was instilled with the non-carcinogenic PAH, pyrene. There is also one group in each of the exposure arrangements which does not receive supplementary treatment.

The following table lists the concentrations of some exhaust components. They are measured in the inhalation chambers and at a distance of a few metres in the ducts feeding the exhaust into the chambers; the values are subdivided into total exhaust and exhaust without particles (Fig.16).

In comparing the measurement values in front of and inside the chambers, it can be seen that the SO_2 concentration decreases on the way to the chambers; this is probably due to adsorption effects. On the other hand, the NO_2 concentration increases along the flow, which is due to the oxidation of NO to NO_2. The total hydrocarbon and methane concentration is higher in the chambers than in front of them. This effect is caused by

	MEASUREMENT			
	IN THE CHAMBERS		BEFORE THE CHAMBERS	
	A	B	A	B
CO [ppm]	16,9 (±3,0)	17,9 (±3,3)	16,3 (±3,2)	18,0 (±3,4)
CO_2 [Vol %]	0,63 (±0,14)	0,67 (±0,14)	0,52 (±0,14)	0,57 (±0,16)
SO_2 [ppm]	4,7 (±1,6)	4,9 (±0,9)	7,9 (±3,8)	7,0 (±1,4)
C_NH_M [ppm]	8,5 (±3,4)	8,9 (±2,8)	5,3 (±3,8)	4,2 (±1,7)
CH_4 [ppm]	2,1 (±0,8)	2,5 (±0,9)	1,0 (±0,33)	1,0 (±0,32)
$C_NH_M - CH_4$ [ppm]	6,3 (±3,0)	6,3 (±2,6)	4,3 (±3,7)	3,3 (±1,4)
NO [ppm]	15,8 (±7,1)	15,6 (±6,9)	17,6 (±8,5)	16,0 (±8,8)
NO_X [ppm]	16,3 (±7,2)	15,9 (±6,5)	17,7 (±10,2)	16,4 (±10,3)
NO_2 [ppm]	0,45 (±0,42)	0,40 (±0,28)	0,10 (±0,09)	0,21 (±0,27)
O_2 [Vol %]	20,0 (±0,7)	19,5 (±0,6)	20,0 (±0,7)	19,1 (±0,5)
PARTICLES [mg/m^3]	-	4,2 (±0,3)	-	-

A: EXHAUST WITHOUT PARTICLES B: TOTAL EXHAUST

Figure 16.

the animals. The particle concentration of 4.2 mg/m^3 corresponds to that of the 1/10 dilution in the 5-month experiment. A comparison with the data of the different exhaust dilutions in the 5-month experiment is impaired, in particular with regard to the soot concentration, because the duct system to the exposure chambers in the life-time experiment is of a different, much larger design which involves additional branching. This latter aspect causes an increased loss of particles on the way to the exposure chambers. It became obvious in the 5-month study, that the particle concentration of the life-time test should not be increased beyond this level. Otherwise effects will become prominent, which are purely caused by the mechanical burden of particles on the lung. In turn, for the dilution chosen, the concentrations of some harmful gaseous substances in the exposure chambers are so low, that they could well be compared with extreme situations of actual environmental conditions.

About 8 months into the life-time study, 22 different clinico-chemical and haematological parameters were measured on those exposure groups, which did not receive supplementary treatment (Fig.17).

INVESTIGATIONS IN CLINICAL CHEMISTRY

ESSAY	CONTROL	GASEOUS EXHAUST	TOTAL EXHAUST
GOT [U/1]	27.9 (±5.7)	32.7 (±6.9)*	34.8 (±5.5)*
GPT [U/1]	52.6 (±9.4)	55.3 (±11.0)	53.6 (±8.1)
γ - GT [U/1]	1.9 (±0.9)	2.0 (±0.68)	4.2 (±1.2)*
CHE [U/1]	289.1 (±33.8)	299.2 (±39.9)	293.9 (±39.8)
LDH [U/1]	170.3 (±43.0)	178.8 (±38.7)	226.6 (±51.8)*
AP [U/1]	206.2 (±43.6)	264.2 (±46.1)*	248.6 (±56.5)*
α - HBDH [U/1]	96.1 (±22.4)	86.1 (±22.1)	106.7 (±21.0)
α - AMYLASE [U/1]	244.0 (±57.2)	277.1 (±60.6)	260.6 (±93.2)
UREA [mg/dl]	50.7 (±16.3)	60.2 (±17.4)*	58.5 (±9.7)*
CHOLESTERIN [mg/dl]	147.7 (±26.2)*	143.6 (±21.6)	127.4 (±20.7)
TRIGLYCERIDE [mg/dl]	127.8 (±49.7)	179.0 (±39.8)*	133.6 (±37.3)
CREATININE [mg/dl]	0.41 (±0.09)*	0.32 (±0.06)	0.30 (±0.05)
PROTEIN [g/dl]	5.7 (±0.35)	6.0 (±0.33)*	5.94 (±0.47)*

Figure 17.

From each group, 25 animals were examined. Enzymes such as GOT, -GT, LDH and alkaline phosphatase were significantly increased, especially in animals exposed to the total exhaust. As in the 5-month experiment, the concentration of blood urea was markedly higher. The total protein content in the serum was also significantly increased in exposed animals. However, the various electrophoretically separated fractions of the serum protein did not differ significantly from the controls.

As in the 5-month experiment, the haematological data showed again that the mean corpuscular volume was significantly higher in the exposed animals, while the erythrocyte and leucocyte counts were again lower in the exposed animals. Whereas the CO-haemoglobin content was still significantly enhanced in exposed animals, the methemoglobin

INVESTIGATIONS IN HEMATOLOGY

ESSAY	CONTROL	GASEOUS EXHAUST	TOTAL EXHAUST
ERY $[10^6/mm^3]$	7.39 (±0.60)	7.04 (±0.48)*	7.07 (±0.62)
LEUCO $[10^3/mm^3]$	5.2 (±1.2)	4.94 (±1.4)	4.25 (±1.1)*
HCT [Vol %]	51.0 (±3.7)	49.7 (±3.3)	49.7 (±4.11)
HB [g/dl]	16.3 (±0.9)	16.8 (±0.76)*	16.0 (±1.27)
MCV $[u^3]$	68.7 (±0.8)	70.3 (±0.74)*	70.1 (±0.91)*
HB_E [pg]	22.1 (±1.3)	23.9 (±1.41)*	22.6 (±1.13)
MCHC [gHb/100 ml Ery]	32.1 (±1.7)	33.8 (±1.97)*	32.2 (±1.60)
CO - HB [%]	0.08 (±0.13)	2.02 (±0.81)*	1.59 (±0.4)*
MET - HB [%]	0.26 (±0.31)	0.35 (±0.38)	0.27 (±0.44)

Figure 18.

values in exposed and control animals showed no difference so far (Fig.18).

These examinations will be repeated every 4-5 months during the experiment, so that at the end of the experiment a statement can be made not only on the histological alterations in the respiratory tract, but also on the general chronic toxicity of diesel exhaust under these exposure conditions.

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CARCINOGENICITY OF DIESEL EXHAUST AS TESTED IN STRAIN 'A' MICE

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ABSTRACT

Groups of Strain 'A' mice were exposed to diesel exhaust by inhalation and diesel particulate by intraperitoneal injection. The animals were exposed from seven to eight weeks and then sacrificed 26-30 weeks postexposure. Other animals were exposed for up to seven months by the inhalation route. Some animals were promoted using urethane at a dose below which tumors would occur. There was no increase in incidence of pulmonary adenomas in the animals exposed to either diesel exhaust or diesel particulate over the control animals. In the animals which were promoted using urethane at a low dose, there was a significant increase in pulmonary adenomas. Diesel pariculate was found in the lungs and bronchial lymph nodes of animals exposed to diesel exhaust 26-30 weeks after cessation of exposure.

INTRODUCTION

The proposed increase in the use of automotive diesel engines in automobiles has caused concern over the potential health effects of exposure to diesel emissions. For the regulated gaseous emissions, automotive diesel engines do not represent an unusual hazard when compared with automotive gasoline engines. However, the particulate emissions from diesel engines are considerably higher than those from automobiles run on unleaded gasoline, and concern has been expressed about the possible hazard of inhalation of the particulate material. Adsorbed onto the surface of the carbonaceous particulate are a number of potentially toxic substances. Among these substances are a number of polycyclic hydrocarbons including known carcinogens such as benzo(a)pyrene (BaP). Extracts of the particulate have been positive for mutagenesis in *in vitro* screening tests. In higher animals additional factors such as particle size, mucociliary clearance, physiological availability of the adsorbed material, residence time and translocation of the particulate from the deep lung all impact upon the toxicity and possible carcinogenicity of diesel particulate. Studies conducted in this laboratory indicate that diesel particulate may remain for a considerable period of time in the lungs of experimental animals following cessation of inhalation exposure (Moore et al, 1978).

In an attempt to assess the carcinogenic potential of diesel exhaust and diesel particulate alone, we chose to use the Strain 'A' mouse lung tumor model first described by Andervont and Shimkin (1940). This model system has been used as a carcinogenic bioassay for numerous chemicals including polycyclic hydrocarbons (Shimkin and Stoner, 1975). The approach used in this study consisted of exposing part of the animals to diesel exhaust by inhalation and the remaining animals to intraperitoneally injected diesel particulate.

METHODS

Diesel-Exhaust Generation System. The diesel exhaust exposure facility is composed of engine system, air dilution system, and animal exposure chambers. The engine system consisted of a six-cylinder (198 cu. in.) Nissan automotive diesel engine and a Chrysler Torque-Flite transmission coupled to an absorption dynamometer. All the exhaust from the engine was mixed with CBR filtered and conditioned air in a dilution tube at a dilution rate of one to 13. From the dilution tube, diluted exhaust entered a large volume mixing chamber from which a portion in Study 1 passes through dynamic flow irradiation chambers (to simulate sunlight) and is then conducted to animal exposure chambers. In Study 2, no irradiated exhaust was used and the exhaust was all diverted away from the irradiation chambers. The remaining exhaust was piped directly from the mixing chamber to the nonirradiated animal exposure chambers. The engine was cycled continuously and run for 20 hours daily in Study 1 and eight hours daily in Study 2. The fuel used was No. 2 diesel fuel.

Continuous cyclic monitoring of the chamber atmospheres was carried out for carbon dioxide, carbon monoxide, total hydrocarbons, and nitrogen oxides. Particulate samples were collected daily. The average concentrations of the measured exhaust components during the exposure period are presented in Table 1 for Study 1 and Table 2 for Study 2. Particulate samples were also collected on Nucleopore membranes for sizing by scanning electron microscopy (SEM). A representative (SEM) photomicrograph of the diesel particulate from both studies is shown in Figure 1.

The particulate material for intraperitoneal injection was collected on Fluoropore filters during Study 1 from the exposure chambers which housed the mice that were being exposed by inhalation. The filters were weighed, scraped, and then sonicated in distilled water to remove the diesel particulate. The suspension was refrigerated, protected from light and resonicated prior to intraperitoneal injection. The particulate was collected and processed one to two days prior to injection. A scanning electron micrograph of the diesel particulate contained in the suspension is shown in Figure 2. For the control, filter samples were taken from the clean air exposure chamber and processed in the same manner as the diesel particulate filters. The suspensions were cultured periodically for bacterial contamination.

Animal Exposure. In Study 1, Strain "A" mice, weighing 19-20 gm each, were obtained from the Strong Research Foundation and the Jackson Laboratory. Male A/Strong mice, were randomly divided into four groups; 25 were placed in the irradiated exhaust exposure chamber, 25 in the nonirradiated exhaust exposure chamber, 25 in the control air chamber, and 25 (positive control) were injected intraperitoneally with urethan (20 mg/mouse) and placed in the control air chamber. The mice were exposed for seven weeks to diesel exhaust and then held in control air chambers for 26 weeks until sacrifice.

For the diesel particulate exposure, the A/Strong mice were divided into six groups of 30 mice each. Groups I, II, III received 235 ug, 117 ug, and 47 ug of diesel particulate per injection, respectively. Group IV (clean air control) received injections of material from the clean air filters. The mice were injected with 0.1 ml of the appropriate solution intraperitoneally three times weekly for eight weeks. Group V (positive control) received an intraperitoneal injection of 20 mg of urethan. Group VI received no injections. The animals were held in control air chambers and sacrificed at the same time as those exposed to diesel exhaust.

The strain A/Jackson mice were randomly divided into three treatment groups of 20 females and 20 male each. One group was exposed to nonirradiated diesel exhaust; one group was

Table 1

Exposure Chamber Atmosphere Concentrations (Study 1)
20 Hours Per Day 7 Days Per Week

Component	Exposure Chambers		
	Non-Irradiated	Irradiated	Clean Air
Carbon dioxide, (CO_2)%	0.252	0.255	0.040
Carbon Monoxide (CO) ppm	15.7	15.4	2.0
Total Hydrocarbons (THC) ppmC			
75°F	15.6	15.0	2.0
350°F	31.2	26.0	---
Nitric oxide, (NO) ppm	5.85	4.94	0.11
Nitrogen dioxide, (CO_2)	2.19	2.73	0.07
Nitrogen oxides (NO + NO_2) ppm	8.04	7.67	0.18
Sulfur dioxide, (SO_2) ppm	2.13	1.91	---
Particulate Mass mg/m^3	6.32	6.87	0.0

Table 2

Exposure Chamber Atmosphere Concentrations (Study 2)
8 Hours Per Day 7 Days Per Week

	Exposure Chamber	
Component	Diesel Exhaust	Clean Air
Carbon dioxide, (CO_2)%	0.29	0.040
Carbon Monoxide (CO) ppm	19.72	2.0
Total Hydrocarbons (THC) ppmC	7.84	2.0
Nitric oxide, (NO) ppm	11.23	0.11
Nitrogen dioxide, (NO_2) ppm	2.65	0.07
Nitrogen oxides $(NO + NO_2)$ ppm	13.88	0.18
Sulfur dioxide, (SO_2) ppm	2.06	----
Particulate Mass mg/m^3	6.39	0.0

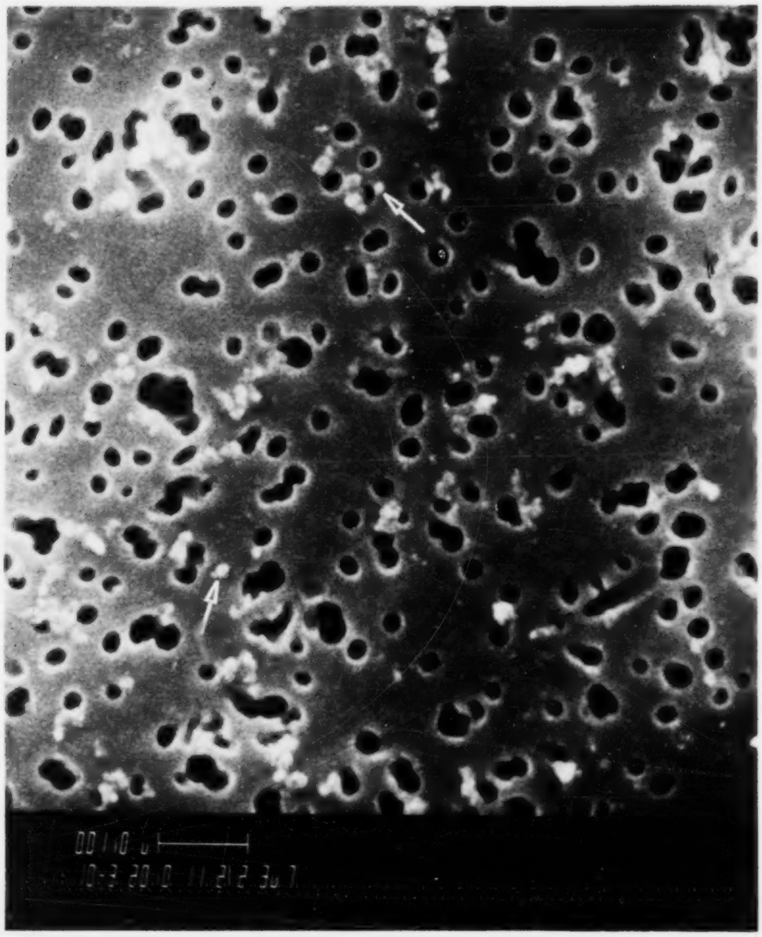

Figure 1 SEM photomicrograph of diesel exhaust particulate. Arrows point to small particulates.

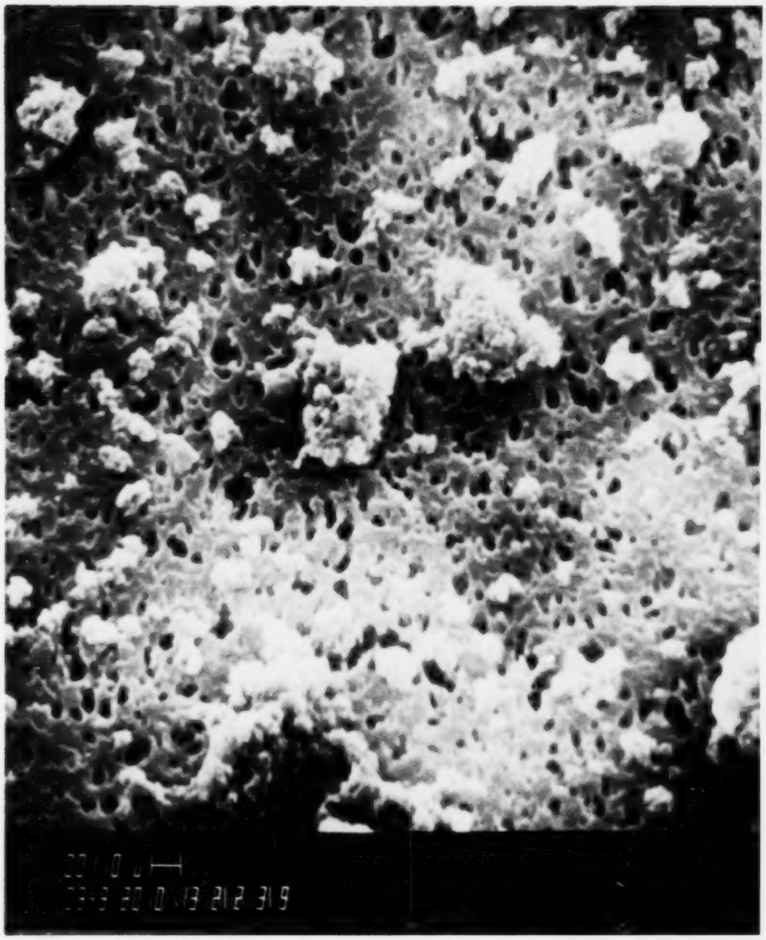

Figure 2 SEM photomicrograph of diesel particulate injected intraperitoneally.

exposed to clean air only; and another group was injected intraperitoneally with urethan (20 mg/mouse) and placed in the control air chamber. The animals were exposed for eight weeks to the diesel exhaust and then sacrificed 30 weeks after cessation of exposure.

In Study 2, 240 female A/Strong mice weighing 18-20 grams were randomly divided into two groups and placed ten animals per cage. One half of each group (60 animals) was given 1 mg urethan intraperitoneally as previously described. The mice were exposed for seven and one-half months in chambers to either raw diesel exhaust or clean control air.

Four additional groups of A/Strong male mice weighing 19-20 grams each were purchased in lots of 200, 220, 240 and 199, respectively. These animals were randomly divided into two groups (raw diesel exposure and clean control air exposure) and placed in their respective exposure chambers. The groups 1, 2, and 3 were exposed until the mice were 36 weeks of age; group 4 was exposed until the mice were 44 weeks of age.

At the time of sacrifice, animals from all groups were killed by an IP injection of an overdose of pentobarbital sodium. The lungs were immediately removed and washed by swirling them in normal saline to remove any accumulated blood or debris from the plural surfaces. The lungs were then placed in Tellyesniczky's fluid (Humason, 1972). Five animals chosen at random from each group had all organs preserved in ten percent buffered formalin for a complete histopathological examination. Any lesions observed in the remaining animals were also preserved for further histopathological examination.

After two to five days the lungs were removed from the Tellyesniczky's fluid and the lobes severed from the primary bronchus. The presence and number of adenomas in the lungs were determined by the presence of milky white nodules. A few typical nodules were selected for histopathological confirmation of the pulmonary adenoma. Any questionable lesions were examined histopathologically for confirmation of the tumor's presence.

RESULTS

Study 1: Diesel Exhaust Exposed Animals. In general, no gross changes in condition or appearance were observed in the mice. During the study several animals died or were found missing in the different groups. However, the number of deaths was not significantly different in the exposed and control groups with the exception of the longest exposure animals (groups 4) See Table 3. The mean body weights of the mice exposed to diesel exhaust are shown in Figures 3 through 10. Exposure to diesel exhaust did not affect the growth rate when compared to the controls.

At necropsy, the lungs from mice exposed to the raw and irradiated diesel exhaust for eight weeks were grey in appearance with numerous dense black areas. The animals exposed for 30 weeks were uniformly black in appearance. On closer gross inspection of the eight week exposed animals, it appeared that the black areas outlined the intermediate and small airways of the lungs. Histopathological examination revealed that macrophages containing large amounts of black granular material were accumulated around the terminal bronchioles. In addition, smaller number of macrophages containing the black granular material could be seen in groups dispersed throughout the lungs (Figure 11). Bronchial lymph nodes were easily identifiable on gross examination because of their color due to the presence of macrophages containing black particles. A section of a bronchial lymph node from a mouse exposed to nonirradiated diesel exhaust is presented in Figure 12. The black deposits are composed of aggregrations of macrophages containing granular diesel particulate material. These macrophages appear to be more concentrated in the medullary spaces although smaller aggregrations may be seen in the cortical areas of the lymph node.

No tumors were observed by gross examination in tissues other than lungs in the diesel exhaust exposed and control air mice; however, two tumors were observed in the skin of mice injected with urethan. The incidence of lung tumors in the mice from each treatment group is given in Table 4. Comparison of the tumor incidence among the different treatment and control groups indicated no significant trend. The A/Jackson mice did not show an increased incidence of tumors in the diesel exposed over the controls. The incidence of tumors in the males was slightly higher when compared to the females, but was similar to the A/Strong mice. In comparing the data, there was no significant difference between the treated and control groups. The incidence of lung tumors in the control group was nearly identical to that obtained in other studies demonstrating the stability of the Strain "A" mice to the induction of lung tumors. In Strain A/Strong mice sacrificed 30 weeks after the first injection, Stoner, et al (1976) reported 31% incidence of lung tumors with an average number of 0.28 tumors per mouse in the untreated controls and a 37% incidence of lung tumors with an average number of 0.42 tumors per mouse in mice injected with 0.85% NaCl solution. All of the 20 mg urethane injected animals had

Table 3

Protocol and Results of Strain A Mouse Diesel Inhalation Tests

Study Designation	C=Control E=Exposed	No. of Survivors Initial No. of Animals	Total Tumors	No. of Animals with 1 Tumor	2 Tumors	3 Tumors	Date Placed in Chambers	Date Removed from Chambers
A-1	C	93/100	20	16	2	0	9/22	5/7
	E	86/100	24	20	2	0	9/22	5/7
A-2	C	104/110	24	18	3	0	10/6	5/21
	E	98/110	18	14	2	0	10/6	5/21
A-3	C	110/120	21	14	2	1	11/14	6/20
	E	105/120	22	16	3	0	11/14	6/20
A-4	C	96/99	27	9	6	2	1/8	10/5
	E	79/100	11	7	2	0	1/8	10/5

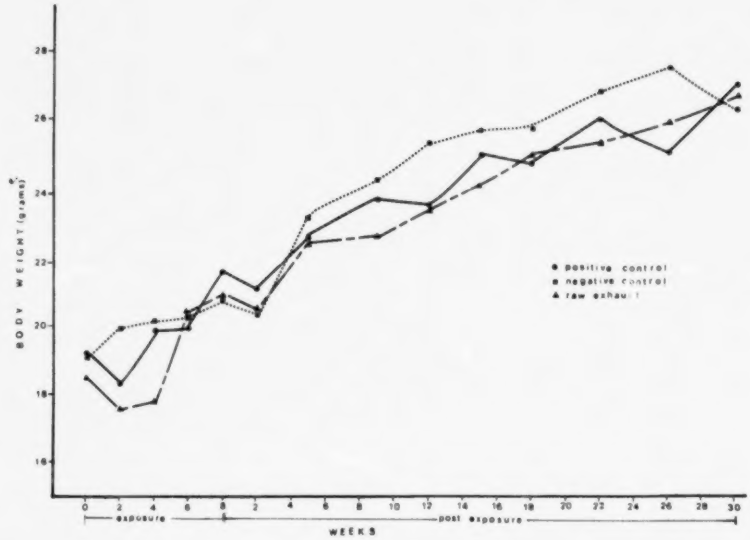

Figure 3 Mean body weights of Strain A/Strong mice exposed to diesel exhaust and controls.

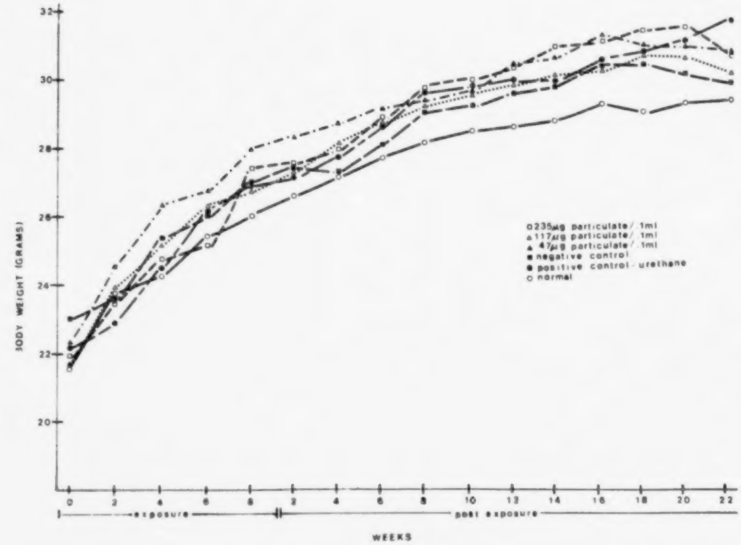

Figure 4 Mean body weights of strain A/Strong mice injected with diesel particulate and control materials.

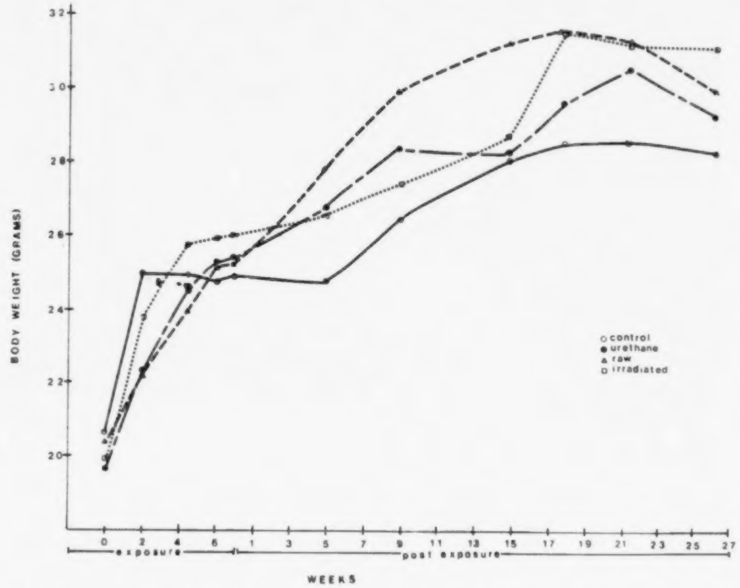

Figure 5 Mean body weights of Strain A/Jackson mice exposed to diesel exhaust and controls.

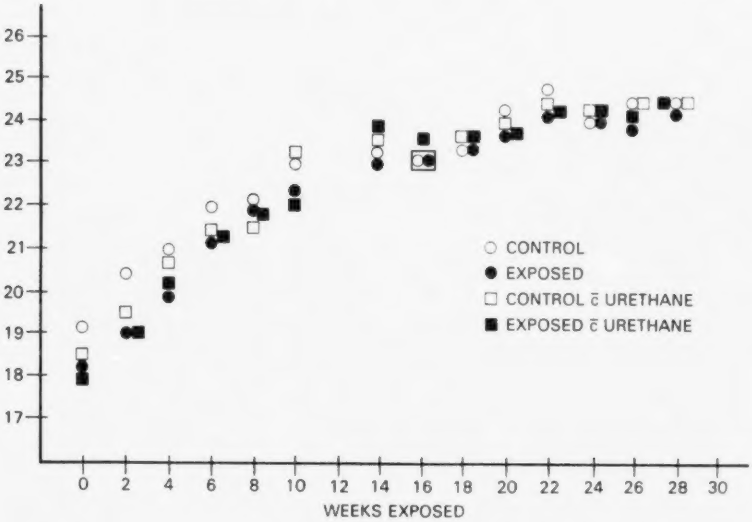

Figure 6 Mean body weights of Strain A/Strong mice exposed in the urethan plus diesel exhaust promotion study.

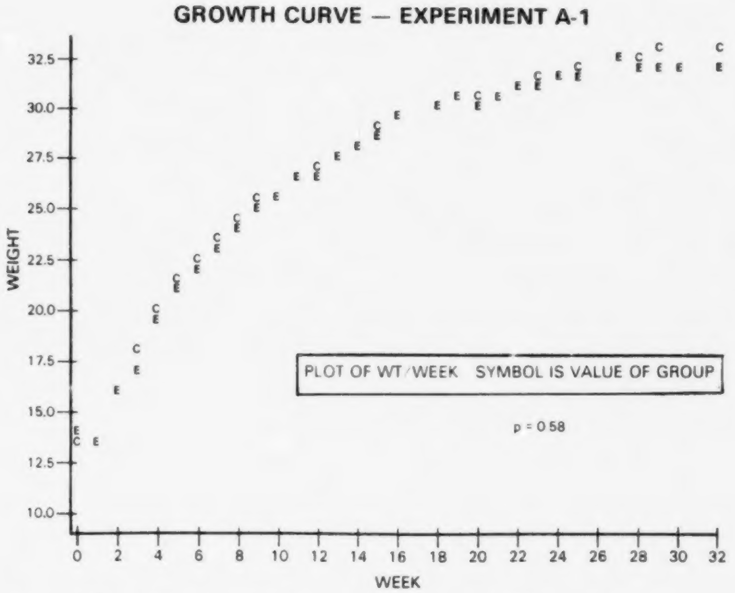

Figure 7 Mean body weights of Strain A/Strong mice exposed to diesel exhaust and controls in Study A-1.

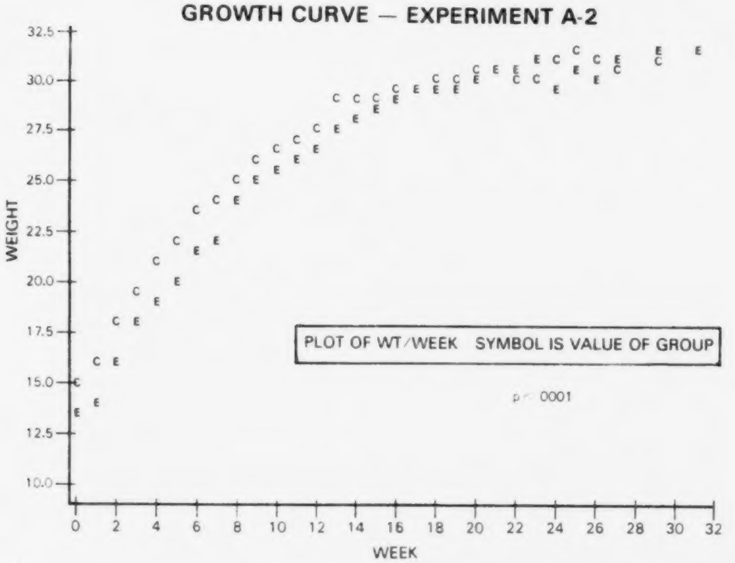

Figure 8 Mean body weights of Strain A/Strong mice exposed to diesel exhaust and controls in Study A-2.

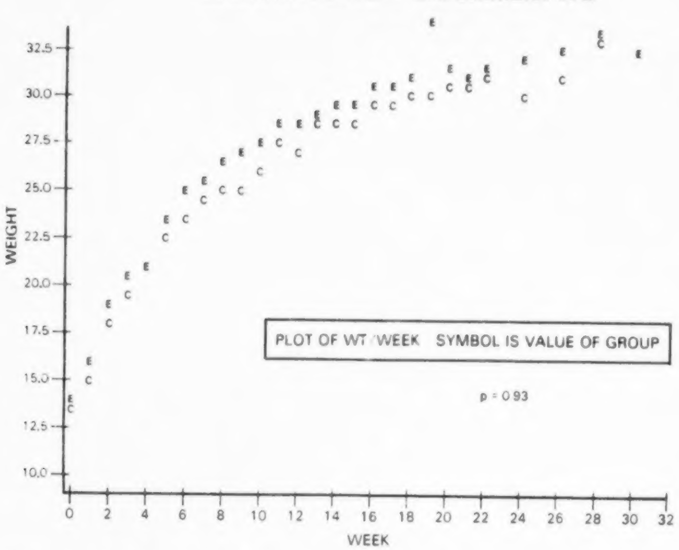

Figure 9 Mean body weights of Strain A/Strong mice exposed to diesel exhaust and controls in Study A-3.

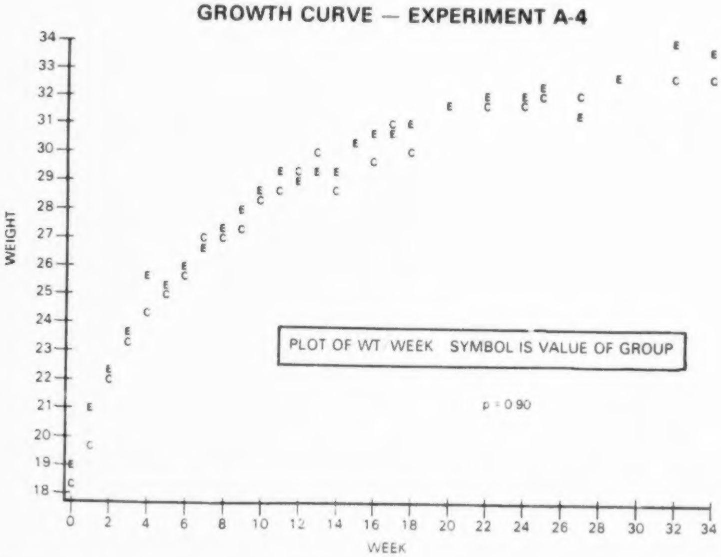

Figure 10 Mean body weights of Strain A/Strong mice exposed to diesel exhaust and controls in Study A-4.

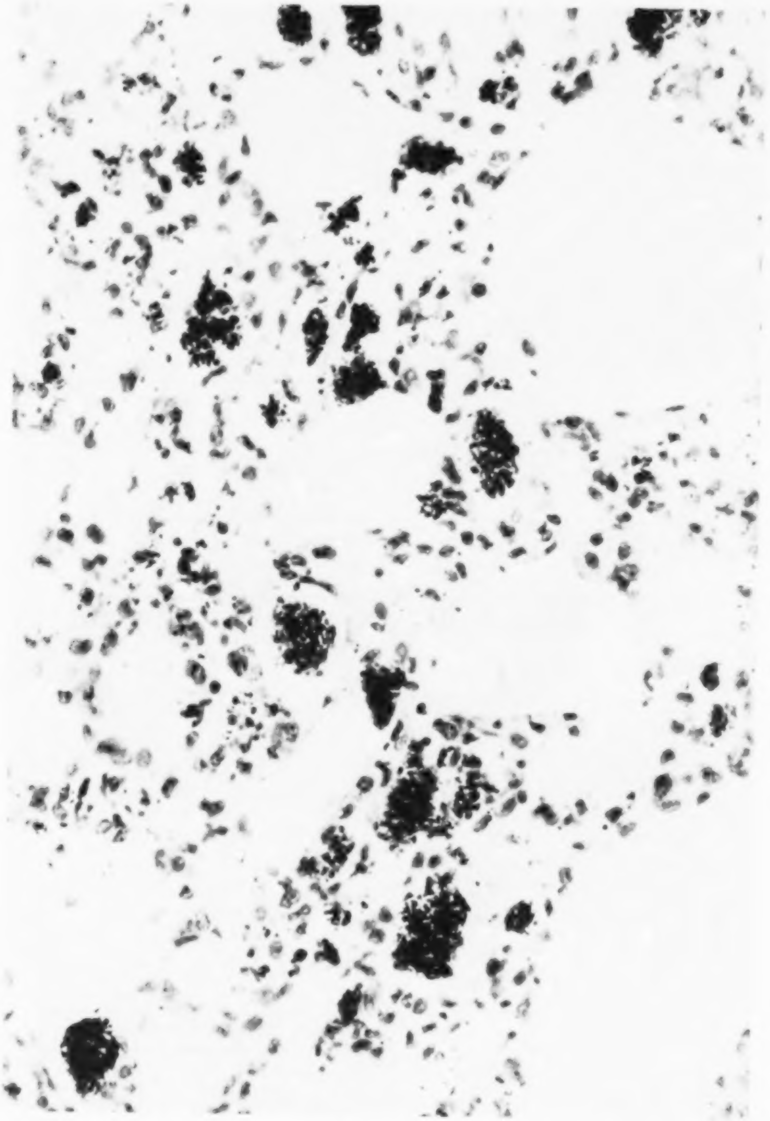

Figure 11 High power photomicrograph showing black granular particulate containing macrophages in alveoli.

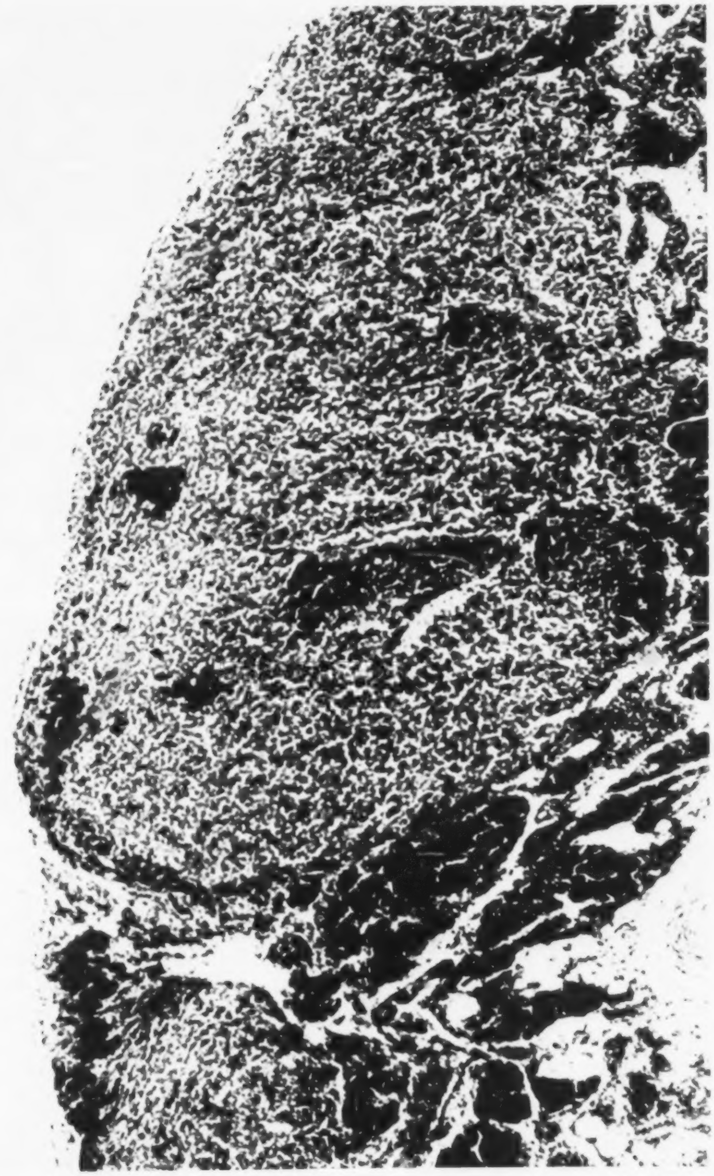

Figure 12 Section of bronchial lymph node from Strain A mice exposed to nonirradiated diesel exhaust. The heavy black material is composed of aggregrations of macrophages containing the granular diesel particulate material.

Table 4

Lung Tumors in Diesel Exposed, Control and Urethan Treated Mice

Treatment		Survivors/Initial	Mice with Lung Tumors	Average No. of Tumors/Mouse
A/Strong Mice				
Nonirradiated exhaust		19/25	7/19	0.63
Irradiated exhaust		22/25	6/22	0.27
Control		22/25	3/22	0.13
Urethan*		23/25	23/23	Multiple
		$X^2 = 2.96$ with 2 df $p = 0.28$		
A/Jackson Mice				
Nonirradiated exhaust	Males	16/20	5/16	0.31
	Females	18/20	6/18	0/50
Control	Males	18/20	5/18	0/33
	Females	18/20	11/18	0.66
Urethan*		17/20	17/17	Multiple
		$X^2 = 5.31$ with 3df $p = 0.150$		

multiple tumor nodules. In some cases, the nodules had coalesced into a large tumor mass making it impossible to determine the number of initial nodules.

Diesel Particulate Injected Mice. Three groups of 30 mice each were injected intraperitoneally with 235 ug, 117 ug and .47 ug particulate, respectively, three times weekly. The highest dose level was chosen to correspond to approximately the inhalation dose assuming 50% retention. Using the diesel particulate concentration of 6 mg/m^3, a minute volume of 0.024 litter and 50% retention of the particulate, the weekly inhaled dose would be approximately 725 ug. The highest dose group received 705 ug/week. There was no evidence of toxicity in any of the treatment groups as indicated by general appearance and growth rate. The mean body weights for the different groups are shown in Figure 4.

The number of tumors in the mice injected with diesel particulate and the control mice is given in Table 5. There was no significant difference between the incidence of tumors in the injected and control mice.

Study 2: The dose of urethane for this group was interpolated from Depaola (1959) as a dose of urethane below which one would expect to find an increase in tumors.

No apparent dose related growth effects or lesions other than lung tumors was apparent. The lung tumor counts are presented in Table 6. The results are significant using the Chi Square test p = .00077.

Male and 30 and 38 Week Exposure. The first three groups designated A1 to A3 showed no dose-related growth effects, gross lesions or microscropic lesions attributed to the diesel exposure. The results of the tumor counts and analysis are presented in Table 3. The fourth group was exposed approximately eight weeks longer and showed a significantly shortened survival time in the exposed groups. The higher mortality occurred in the final phase of the exposure and was thought to be the result of lessened resistance to disease.

The randomly chosen animals for histopathological examination showed no diesel-related lesions which could be attributed to exposure. The lungs, except for questionable nodules were not routinely examined for histopathological lesions.

Table 5

Incidence of Lung Tumors in A/Strong Mice Injected with Diesel Particulate, Urethan and Controls

	Treatment (Total Dose)	Survivors/ Initial	Mice with Lung Tumors	Average No. of Tumors/Mice
Group I	5.6 mg	30/30	8/30	0.30
Group II	2.8 mg	30/30	10/30	0.33
Group III	1.1 mg	30/30	2/30	0.06
Group IV	injected control	30/30	8/30	0.33
Group V*	20 Mg urethan	29/30	29/29	multiple
Group VI	untreated control	28/30	7/28	0.25
	$X^2 = 6.6809$ with 4df $p = 0.154$			

Table 6

Survivors and Number of Tumors in the Control and Urethan-Diesel Exposed Groups of Animals

Treatment	Survivors / Original Number	% Survivors	No. of Animals with Tumors	Number of Tumors
Control	58/60	93.5	4	5
Control + Urethan	52/60	83.8	9	13
Exposed	56/60	90.3	14	18
Exposed + Urethan	59/60	95.2	22	23

DISCUSSION

Generally, the results of these studies are negative with the exception of the urethane injected exposed animals (promotion study). Even though the control animals' tumor rate is below that which is expected for Strain "A" female mice, the hypothesis that the test is positive can be tested by substituting the known tumor value for our Strain A/Strong control mice. This comparison is shown in Table 7. These latter control mice are males, whereas the promotion animals are all females. The literature references report the lower spontaneous rate occurs in females which would tend to make the test more significant.

The Strain "A" mouse lung tumor model has been used by several investigators as a bioassay for assessment of carcinogenicity of polycyclic hydrocarbons. Shimkin and Stoner (1975) summarized the findings on ten polycyclic hydrocarbons and related compounds following a single intravenous injection of approximately 0.25 mg of aqueous dispersions with sodium sulfosuccinate as a wetting agent. Some of the polycyclic hydrocarbons such as 3-methylcholanthrene, dibenz(a)anthracene and benzo(a)pyrene were potent carcinogenic agents while benz(a)anthracene and 5-methoxy-7-propyl-benz(a)anthracene demonstrated no activity. In these tests ten to 19 animals were used for each compound to detect the tumor incidence. In our experiments no increase in the incidence of lung tumors was found following as much as nine months of inhalation exposure to diesel exhaust alone, however, the addition of urethane in low doses did elicit a positive response to the combination of urethane and diesel exhaust. The role of urethane as an initiator and/or promotor for lung cancer is known. In the case of our promotion study, neither the urethane exposure or diesel exhaust exposure alone produced a significant increase in lung nodules. The two exposures together, however, did produce a weakly positive significant increase in pulmonary adenomas in the Strain "A" mouse. Diesel particulate was present in the lungs and draining lymph nodes 26-30 weeks after cessation of exposure. The duration of the polycyclic hydrocarbons in the lungs after cessation of exposure cannot be ascertained because the elution rate of this material off the surface of the diesel particle under physiological conditions has not been determined. There is little information on the factors governing release or biotransformation of the polycyclic hydrocarbons by cells phagocytizing the particulate material.

Table 7

Comparison of Urethan Promoted Diesel Exposed A/Strong (Female) Mice and Historical Controls (Male)

Group	Survivors Initial	Mice with Lung Tumors	Chi Square Test Groups[1] p value
1 Control	58/60	4	Group 1, 2, 3, 4 p = .00077
2 Control + Urethan	52/60	9	2, 3, 4, 5 p = .0021
3 Exposed	56/60	14	2,5 3,5 p - .92 p = .21
4 Exposed + Urethan	59/60	22	3,4 2,4 p = .16 p = .019
5 Historical Controls	60/60[2]	10	

[1] Due to performing several tests on these data significance should be at the p - .01 level.

[2] This figure represents a portion of historical controls and is not representative of the total number or of the percentage of survivors.

The presence of the particulate material in the lymph nodes indicates the transport of diesel particulate from the alveoli to the interstitium and the lymphatics. The mechanism of transport from the alveoli to the interstitium is most likely the same as shown by Adamson and Bowden (1978) who have demonstrated the presence of carbon within sessile macrophages of the hilar lymph nodes 12 hours following intratracheal instillation of carbon in mice. They found migration of free carbon particles through the type 1 cells to the interstitium where they were rapidly phagocytosed by interstitial macrophages. Also the presence of carbon in hilar lymph nodes 12 hours before appreciable amounts of carbon had been taken up by interstitial macrophages, suggest that carbon can reach the lymph nodes in the free state. It still remains to be determined if the diesel particles can serve as a mechanism of transport of polycyclic hydrocarbons to other tissues.

The method used to generate exhaust and inhalation route of exposure correspond closely to the human potential for inhalation of diesel emissions. The exposure generation system used in this study is probably as close to environmental reality as can currently be designed for studying such emissions. The concentration of the particulate in the exposure was several orders of magnitude higher than would normally occur under severe environmental conditions and the clearance mechanisms of the lung were obviously able to remove only part of the diesel particulate as indicated by the presence of the material in the lungs 26-30 weeks after cessation of exposure. In vitro tests of extracts of diesel particulate emissions have indicated microbial mutagenicity. The significance of an increased incidence of tumors in these mice underlines the need of animal inhalation exposures when trying to assess environmental risk to humans.

Reznik-Schuller and Mohr (1977) demonstrated the development of pulmonary adenomas in Syrian Golden hamsters after intratracheal instillations with automobile exhaust condensate. They pointed out that the high tumor rate may, in part, be due to the failure of the mucociliary defense mechanism to clear the airways of a large portion of the condensate. Further, they indicated that the instillation procedure does not correspond to the human situation of inhalation and that the instilled material was administered as a condensate, whereas most of the inhaled substances are gaseous, or bound to smaller carrier particles which are likely to be removed through mucociliary action. An increased incidence of skin tumors has been found by some investigators (Kotin et al, 1955) following skin painting of diesel extracts. This study illustrates the necessity of continued investigation in order to fully assess the carcinogenic potential of atmospheric carcinogens. Although initially negative by the inhalation route diesel exhaust, when potentiated with a promotor/initiator, demonstrated a positive response. There are numerous carcinogens and cocarcinogens in the environment and the possibility of interaction is present.

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General Discussion

G. STONER: During a 36-week period if you inject one milligram of 3-methyl cholathene per mouse, you get 40 to 50 lung tumors per mouse. With this dose of urethane you usually get one tumor per milligram and with 20 mgs of urethane you get about 20 tumors per mouse. If diesel exhaust is active at all, it is extremely weak in this system. I suggest that you conduct some experiments where you keep the animals around for a little longer period of time because some of the weaker agents require a longer time period to produce a tumorigenic response. This would permit you to pick up some weak positive responses which you won't get in a 36-week period.

C. RUDD: Intraperitoneal injections intrigue me. When you suspend the diesel particles in distilled water, do the diesel particles stay on the top or are they in suspension? How do you succeed in injecting it?

J. ORTHOEFER: The diesel particulate at the concentration we had does not stay in suspension. In order to achieve an accurate injection dose we agitated the particulate constantly with magnetic stirring bars. We then pour about 0.5 ml into a syringe and inject each of five mice with 0.1 ml/mouse. By continuing the stirring motion there was no problem with keeping it in suspension.

C. RUDD: What size needle did you use?

J. ORTHOEFER: A 25 gauge needle was used for the IP injection.

C. RUDD: From IP injection, would you suspect that the diesel particulates travel to the lung or does the organic matter leach off the particle?

J. ORTHOEFER: The cytopathology of the internal organs show massive amounts of the carbon particles throughout the abdominal cavity and lymph nodes. It is possible that some material may leach off the particle and enter the bloodstream to be carried to the lungs.

Session VIII

EPIDEMIOLOGY STUDIES INVOLVING HUMAN EXPOSURE TO DIESEL EMISSIONS

Chairman:

Robert Waller

A Review of the Literature: Human Health Effects Associated with Exposure to Diesel Fuel Exhaust.
Calabrese, Edward J., Gary S. Moore, Ruth Ann Guisti, Carol A. Rowan, and Elizabeth N. Schulz.

Trends in Lung Cancer in London in Relation to Exposure to Diesel Fumes.
Waller, R. E.

Human Data Associated with Diesel Exhaust.
Lachtman, Dennis S.

A Retrospective Cohort Study of Diesel Exhaust Exposure in Railroad Workers: Study Design and Methodologic Issues.
Schenker, Marc B., M.D., and Frank E. Speizer, M.D.

Characterization of Diesel Exposure Groups.
Hansknecht, Donald F., Richard A. Ziskind, and Michael B. Rogozen.

An Industrial Hygiene Characterization of Exposures to Diesel Emissions in an Underground Coal Mine.
Wheeler, Robert W., P.E., Frank J. Hearl, and Michael McCawley.

A REVIEW OF THE LITERATURE: HUMAN HEALTH EFFECTS ASSOCIATED WITH EXPOSURE TO DIESEL FUEL EXHAUST

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While the need to assess the potential impact of diesel fuel exhaust on human populations is now recognized as an important task because of the projected increase in the use of this fuel, the epidemiological data upon which to base an assessment are quite limited. There have been nine major epidemiological studies which have attempted to evaluate the influence of diesel fuel exhaust on human populations. In all cases, these studies have dealt with occupational exposures to diesel fuel exhaust. This is of concern since a prime consideration is the influence of diesel exhaust on the general public as a result of ambient, not occupational exposures.

These studies have covered a broad range of occupations in which exposures may occur to diesel fuel exhaust including bus and railroad mechanics, coal miners, salt, iron, and potash mine workers. These studies have been conducted in several different countries - England, Egypt, Sweden, and the United States by a variety of research teams. Health outcomes considered include deaths due to lung cancer or other causes; morbidity due to pneumonoconiosis, bronchitis; asthma; influenza; and respiratory infection. Other non-specific indicators of pulmonary function were included such as reduced pulmonary function test values, and reporting of symptoms such as cough, wheezing, shortness of breath, and phlegm production. All the studies reviewed were conducted between 1956 and 1978.

Lung Cancer and Morbidity and Mortality Due to Other Causes

Three of the studies reviewed considered lung cancer mortality or mortality from other causes as a health end point (Raffle (1), Kaplan (2), Waxweiler et al (3)). A major and unavoidable limitation to these studies is that widespread dieselization of transportation and mining operations did not generally occur until the 1950's or even later in some instances. This resulted in a total industrial exposure period to diesel exhaust emissions of from only several years to generally not more than 25 years depending on the study. Thus, there may not have been enough time to detect a significant increase in lung cancer mortality due to extended latency periods.

Raffle (1) found no excess lung cancer mortality in a study of diesel exposed London Transport bus mechanics aged 45 to 64 between 1950-1954. Several methodological limitations must be considered when interpreting the findings of this study.

There is evidence that smoking habits may have differed between the highly exposed group and their occupational controls as diesel exposed garage workers were not permitted to smoke on the job while some petrol-fueled buses were still in use (4). No control for the smoking habits of the workers was attempted. Also, diesel exposure was not well documented. Commins, et al. (5), reported on the analysis of air samples from two of the London Transport garages considered to have relatively poor ventilation. These analyses conducted during the follow-up period indicate that the exposure to diesel components in these two garages may not have differed substantially from the general ambient levels to which the occupational and general population control groups were exposed to at this time.

While the lack of consideration of smoking differences, and the apparent absence of marked differences in respiratory carcinogen exposure between the diesel exposed workers and control groups seemed to bias against finding any lung cancer differences, the Raffle (1) study design tried to compensate by comparing deaths, retirements and transfers due to cancer among the mechanics to only deaths from lung cancer in the general population, thereby slightly increasing the number of diesel related lung cancer cases in several comparison groups. However, this procedure did not result in any marked change in the annual rate of lung cancer cases.

Kaplan (2) also found no association between the level of occupational exposure to diesel exhaust and lung cancer deaths in his study of railroad workers. Again, there was no control for smoking habits of workers and the individual exposure to diesel exhaust was not well documented.

Waxweiller, et al. (3), found no significant mortality differences between diesel exposed and non exposed potash miners. Causes of death considered include (1) tuberculosis, (2) malignant neoplasms, (3) influenza and pneumonia, and (4) "other respiratory diseases" i.e. bronchitis, pneumonconiosis etc. The maximum diesel exposure in this study, however, was only 17 and 10 years, respectively, depending on the group of workers considered and individual exposure, length of employment and previous occupational exposure are not controlled for.

Respiratory Morbidity

Limited research has assessed the potential relationship of exposure to diesel exhaust and specific types of morbidity including bronchitis, asthma, influenza, and respiratory infection. These health effects would have great significance for high risk segments of the population such as the very young and the elderly. Unfortunately, the two studies which attempt to address this issue El Batawi and Nowier (6), and Jorgensen and Svensson (7) are not very helpful with respect to evaluating the potential health effects of diesel exposure because of problems in study design and execution. El Batawi and Noweir (6) found a higher frequency of upper respiratory tract disease, bronchitis, asthma, peptic ulcers, gastritis, and high blood pressure in a survey of Alexandria diesel bus mechanics than had been reported in studies of other occupational groups. However, the lack of a comparison group and the extremely high proportion of heavy smokers and night shift workers makes it difficult to assess the effects of the diesel exposure. Jorgensen and Svensson (7) examined rates of bronchitis and respiratory infection among diesel exposed iron miners. While the data seem to indicate a synergistic effect of smoking and exposure to diesel mine environments, the exposure to diesel exhaust cannot be separated from the effect of exposure to the mine environment as a group of surface mine workers was used as a control.

Respiratory Symptoms and Pulmonary Function

The body of the literature suggests an association of diesel exposure, reduced pulmonary function and increased reporting of non specific pulmonary complaints. Reger and Hancock (8), in a large study of over 700 diesel exposed coal miners and their matched non diesel exposed coal miner controls, found significantly more diesel exposed miners who reported symptoms of persistent cough, phlegm, and exacerbation of cough and phlegm. Pulmonary function was generally found to be poorer among exposed workers, and the disparity of symptoms and pulmonary function between exposed and nonexposed workers was found to increase with the length of employment. However, decreases in pulmonary function did not seem to be consistently related to the level of diesel usage in the diesel mine and some complaints. However, wheezing and shortness of breath were reported more frequently among the control miners. Additionally, diesel exposure is compounded here as in all the other mining studies with exposure to the mine environment, and the similarity of the diesel and non diesel mines on factors other than diesel usage is not well documented. It should be mentioned that the control group was drawn from a group of miners evaluated nearly a decade prior to assessment of the diesel exposed workers (9). While selection of such a control group is not inappropriate, it does present several methodological questions which may affect the outcome of the study. For example, while individuals were carefully matched for smoking status, it is possible that the cigarettes smoked in 1969, at the time of the aquisition of the control group data, contained on average fewer filtered brands with more tar and nicotine so as to have differentially affected the controls. Also, because the exposed group and controls were evaluated in different studies, years apart, differences in technique and in coaching effect may have resulted in a lack of uniformity of data collection between the two groups.

In a subsequent study, Reger, Hankinson, and Merchant (10) examined a subset of 60 diesel exposed workers from the matched pair study. These workers were selected to represent a broad spectrum of mine jobs. Fifty-five non exposed mine workers were population matched to the exposed workers and changes in pulmonary function from the beginning to the end of the work shift were examined. While the absolute decreases for the five pulmonary function parameters considered were consistently greater for the exposed miners, none of the between-group differences were found to be significant. Population matching was imperfect and resulted in an older control group with a longer mean underground exposure. While age and length of occupation were not found to be directly associated with ventilatory function, a more complex relationship could exist.

Gamble, et al. (11), examined the relationship between the level of exposure to NO_2 and changes in pulmonary function over the course of the work shift in from five salt mines in the U.S. of which four used diesel equipment to varying degrees. Levels of NO_2 were found to be directly related to the change in pulmonary function for four of five pulmonary function tests considered. The level of respirable particles was not found to be associated with pulmonary function changes. Battigelli, et al. (12), reported no significant differences in pulmonary function or respiratory complaints (coughing, phlegm, dyspnea, etc) between diesel exposed railroad house workers and non exposed yard workers. Smoking was not well controlled for and controls were found to have a higher smoking index (packs/day x years smoked) than did the exposed group. An additional potential source of bias is the large number of unmatched exposed workers included in analysis. No information is given as to the similarity of these unmatched exposed workers to their unmatched counterparts.

The long term significance of decrements in pulmonary function and of non specific respiratory complaints is not clear. Gamble, et al. (11), suggest that decrements in pulmonary function may be early indicators of increased susceptibility for irreversible lung pathology. A longitudinal follow-up of these studies would be of great value in elucidating this question.

In summary, the existing literature indicates:

1. No evidence of excess cancer mortality due to exposure to diesel fuel exhaust. However, existing studies must be interpreted with caution in light of methodological limitations specific to each study. In fact, since the three lung cancer published studies (1)(2)(3) do not contain sufficient information on diesel exhaust exposure characterization, differential patterns of smoking history, as well as having latent periods often much shorter than 25 years, it is not possible to adequately assess the hypothesis that diesel exhaust is an etiologic agent in respiratory cancer.
2. Insufficient evidence to evaluate the relationship between exposure to diesel exhaust and other lung disorders, including bronchitis and respiratory infection.
3. Some evidence to suggest that exposure to diesel exhaust may be associated with reduced pulmonary function. The long term significance of this reduction has not yet been evaluated.

Clearly, more research is needed to investigate the potential long term and immediate health effects of exposure to diesel exhaust in the occupational environment. Additionally, research must be conducted to investigate the potential health effects of exposure to diesel exhaust in ambient air on the general public. Previous studies have dealt with occupational groups in which workers tend to be self-selecting and therefore may not be at comparable risk to adverse health effects than the general public. Before accurate risk assessments are derived, those groups likely to be at high risk to the potential adverse health effects of diesel exhause must be identified and fully evaluated.

Following is a summary table of the human health effects studies which illustrates study design, exposures, health endpoints examined, and results, as well as comments on the study strengths and weaknesses. In addition, the detailed assessment of these studies, a 70 page document, will be available through EPA.

SUMMARY OF HUMAN HEALTH EFFECTS STUDIES

Author	Study Design	Exposure Type	Population Studied	Health Endpoints	Results	Strengths and Weaknesses
Transportation workers						
1. Raffle (1957) (London) (1)	Cohort Type*	Diesel Bus Exhaust	Exposed - London Transport Staff Garage Mechanics - Males Aged 45-64	Deaths, retirements or transfers due to lung cancer	Found no excess mortality from lung cancer among garage workers after 5 years of follow-up. Unpublished results of 25 year follow-up confirm these results.	1) High exposure group may not in fact have received an exposure significantly greater than the groups classed as low exposure or than the general public. 2) Follow-up time not sufficient to detect excess mortality if latency period for lung cancer exceeds 25 years. 3) No control for smoking habits. High exposure group had on the job smoking restrictions. This could reduce lung cancer rates in the high exposure group. 4) Since the study only included men up to 64 years of age, any cancers that may have realistically occurred over this age group were eliminated from the study.
2. El Batawi & Noweir (1966) (Alexandria, Egypt) (6)	Survey	Diesel Bus Exhaust	Male day, night and alternating shift workers at two bus maintenance garages in Alexandria, Egypt. (N = 161)	Upper respiratory tract diseases, chronic bronchitis, asthma, peptic ulcer, gastritis, high blood pressure	Higher frequency of health end points than expected on the basis of other occupational groups. However, results are uninterpretable with respect to diesel exposure due to the nature of the study design.	1) Survey design provides no controlled basis for evaluating the association of exposure to reported outcomes. 2) No control for smoking habits. 72% of the surveyed workers were reported to be heavy smokers. 3) No control for 'high stress' conditions. In examining stress related outcomes 75% of workers were regular night or alternating shift workers.

*The same group of people who have not consistently been studied since, in progressive years, additional workers were added to the study.

SUMMARY OF HUMAN HEALTH EFFECTS STUDIES (Continued)

Author	Study Design	Exposure Type	Population Studied	Health Endpoints	Results	Strengths and Weaknesses
3. Battigelli, Mannella and Hatch (1964) (United States) (12)	Cohort	Diesel Railroad Engine Exhaust	Exposed railroad house workers exposed to high levels of diesel exhaust in routine maintenance procedures. (N = 210) Non-exposed non diesel exhaust exposed railroad yard workers matched to exposed workers on age, body size, and extra-pulmonary medical history. (N = 150)	Pulmonary function tests and frequency of nonspecific respiratory complaints: cough, phlegm, dyspnea, etc.	No significant between group differences were found in pulmonary function or in reported respiratory complaints. The data suggest a synergistic interaction between smoking and exposure to diesel exhaust.	1) Smoking habits not well controlled for. Smoking index (packs/day x years smoked) higher for control group. 2) No control for previous occupational exposure for control group. 3) A large number of unmatched exposed workers are included in the analysis. This represents a potential source of bias if the unmatched exposed workers on factors related to the outcomes considered.
4. Kaplan (1959) (United States) (2)	Cross-sectional Type*	Diesel Railroad Fumes	Railroad workers (N = 6506) classified by their level of exposure to diesel exhaust into 3 groups: 1) Workers routinely exposed to diesel exhaust. 2) Workers with less occupational exposure to diesel exhaust. 3) Workers with little or no occupational exposure to diesel exhaust.	Deaths attributable to lung cancer	No association between level of exposure and cancer deaths.	1) Follow-up time may not have been sufficient to detect excess lung cancer mortality if the latent period exceeds 25 years. 2) No control for smoking habits. 3) Actual occupational exposure levels are not well documented.

*Study group was compared to the general population for expected lung cancer death rates and death rates from all causes.

SUMMARY OF HUMAN HEALTH EFFECTS STUDIES (Continued)

Author	Study Design	Exposure Type	Population Studied	Health Endpoints	Results	Strengths and Weaknesses
Mine Workers						
a) Coal Miners						
1. Reger & Hancock (United States) (8)	Cohort	Diesel Mine Machinery and Coal Dust	Exposed - underground coal miners exposed to diesel machine exhaust. (N = 722) Non-exposed - underground miners not exposed to diesel exhaust individually matched to exposed miners for geographic area, smoking, age, race, height and tenure of occupation (N = 722) selected from the Interagency Study of Coal Worker's Pneumoconiosis.	Indices of pulmonary function 1 - spirometric testing 2 - radiologic examination for pneumoconiosis 3 - MRC-respiratory questionnaire	Significantly more exposed miners reported symptoms of persistent and exacerbated cough and phlegm. Pulmonary function was in general poorer among exposed workers. The disparity of symptoms and pulmonary function between exposed and non-exposed workers increased with tenure of occupation. In contrast symptoms of dyspnea and wheezing were found to be significantly more common among the non-exposed workers.	1) The study design and matching procedures give this study a high degree of internal validity. 2) Diesel exposed miners and their matched control were, however examined in two different studies introducing the possibility of bias due to differential recording and measurement of data. 3) Exposure to diesel exhaust is confounded by the simultaneous exposure to coal dust. Exposures of exposed and non-exposed workers to non diesel particulates are not well documented.
2. Reger, Hankinson & Merchant (United States) (10)	Cohort	Diesel Mine Machinery and Coal Dust	Exposed - a sample of 60 workers selected from the above exposed worker group to represent the spectrum of mine jobs. Non-exposed - 55 underground coal workers not exposed to diesel fumes. Population matched for smoking status, age, length of employment and geographic location.	Changes in pulmonary function tests over the course of a work shift.	While the absolute decreases for the five ventilatory function tests considered were consistently greater for the exposed workers, none of the between group differences was significant.	1) The population matching is imperfect and results in an older control group with a longer mean underground exposure. However, age and length of exposure were not found to be directly related to ventilatory function. The association could be more complex and other cumulative indices of exposure should be considered. 2) Since the exposed group was drawn from a group of exposed miners later shown to have a significantly reduced pulmonary function, an inherent bias may have been introduced.

SUMMARY OF HUMAN HEALTH EFFECTS STUDIES (Continued)

Author	Study Design	Exposure Type	Population Studied	Health Endpoints	Results	Strengths and Weaknesses
b) Non-Coal Miners						
1. Gamble, Jones Hudak and Merchant (United States) (11)	Cross-sectional	Diesel Mine Machinery and Salt Dust	Salt miners exposed to varying levels of diesel exhaust. (N = 246)	Changes in pulmonary function over the course of a work shift.	NO_2 was found to be directly related to the change in pulmonary function over shift for four of the five pulmonary function tests considered. The level of respirable particulates was not found to be significantly related to changes in pulmonary function.	1) Appears to be a methodologically sound, well-controlled and thorough study. 2) NO_2 was not proven to be generated from diesel equipment and may have been produced by explosives.
2. Waxweiller Wagoner, V.E. & Archer (1973) (3)	Historical Cohort	Exposure to Potash Mine Environment and Diesel Exhaust	Exposed - Male (N = 2743) underground potash miners who worked at least one year between 1940 and 1967. a) Workers from two dieselized mines exposed additionally to diesel fumes. b) Workers not from diesel mines. Non-exposed - (N = 1143) Potash millers-surface workers not exposed to mine environment.	Mortality ratio for various causes of death relative to the age, sex, calendar time, race and cause specific mortality rates for the general U.S. population causes considered include: 1) Tuberculosis 2) Malignant neoplasms 3) Influenza & pneumonia 4) Bronchitis, pneumoconisis and other respiratory diseases.	No significant mortality differences between diesel exposed and non diesel exposed miners.	1) Exposure to diesel exhaust is not quantified nor is length of employment in diesel mine or previous diesel exposure controlled for. 2) Maximum diesel exposure in the mines studied was only 17 years. This length of time may not be sufficient to detect an increase in mortality for neoplastic disease. 3) Actual diesel exposure is not well-documented.
3. Jorgenson & Svensson (1970) (Sweden) (7)	Cohort	Exposure to Iron Mine Environment and Diesel Exhaust	Exposed - 120 randomly selected underground iron mine workers exposed to diesel exhaust. Non-exposed - 120 randomly selected surface workers.	Bronchitis, respiratory infection and decrease in pulmonary function tests over a work shift.	Results are uninterpretable with respect to diesel exposure due to the nature of the study design.	1) Health effects of exposure to diesel exhaust cannot be separated from health effects of exposure to the mine environment.

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General Discussion

J. VOSTAL: You have mentioned that you would like to differentiate between occupational exposures and nonoccupational exposures, and you have stated that it could be influenced by a self-selection process by the workers. Since the most important question we are addressing concerns the lung, could you be more specific as to how anyone could be preselected to lung cancer susceptibility.

E. CALABRESE: I am not sure that the most important question is that of respiratory cancer. From my own prospective, respiratory cancer is only one factor to consider along with a whole host of respiratory disorders, in terms of the effects of diesel exhaust on individuals, including bronchitis, etc. At the present time I think these should enjoy an equally important concern. With regard to a self-selected group for detection of respiratory cancer, that would be a difficult, if not impossible, situation. Regarding occupational epidemiological studies, the concept of the healthy worker effect results from either self-selection or selection by the occupational physician at employment. In the latter case individuals looking for a job might be turned away because they may be carrying an identifiable type of disease process. Preselection may be very effective for respiratory disorders or some other type of illness but usually it is more apparent with regard to death by circulatory disorders. The healthy worker effect is not usually as large with respect to cancer, but can be a very important factor in regard to noncancer illness.

TRENDS IN LUNG CANCER IN LONDON
IN RELATION TO EXPOSURE TO DIESEL FUMES

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ABSTRACT

Mortality from lung cancer among males in England and Wales is now beginning to decline, and the urban excess that has persisted for many years is also becoming smaller. There is nothing in these [illegible]onal trends to suggest that pollution from diesel vehicles, which has been increasing in recent decades, would be involved. Some preliminary results are presented from a long-term study of the incidence of lung cancer among London Transport staff, including men who work in bus garages where there are high concentrations of smoke from diesel buses. Over the 25 year period from 1950-74, the numbers of cases reported in each of the several job categories have been below those expected on the basis of greater London rates. The absence of data on smoking habits makes it difficult to determine whether the small differences in rates between job categories are of any importance, but the standardized mortality ratios are all well within the range of those found in national studies of lung cancer mortality and smoking in relation to occupation.

For many years there has been concern about the possible influence of exposure to fumes from motor vehicles on the incidence of lung cancer. This arose partly as a result of the demonstration of carcinogenic comounds such as benzo(a)-pyrene in urban air (Waller, 1952) and specifically in emissions from petrol and diesel engines (Kotin et al., 1954, 1955). In the case of diesel engines, emissions of such compounds are applicable only under poor operating conditions, when there is much smoke, and it is clear that at least prior to the implementation of the Clean Air Act, coal fires were more important sources of benzo(a)pyrene in British towns than motor vehicles (Lawther and Waller, 1976). With the gradual elimination of coal smoke in London (and in most other major cities) over the past 25 years, concentrations of benzo(a)pyrene in the air have fallen dramatically, as illustrated in Table 1.

It is difficult to determine from an examination of trends in lung cancer mortality in the general population whether any component of air pollution may be involved in the actiology. Figure 1 shows the age-specific death rates for lung cancer among males in England and Wales arranged according to date of birth. Rates at each age increased steeply in relation to date of birth up to the beginning of the century, but men born since about 1911 have been experiencing slightly lower death rates than their predecessors. While the substantial fall in smoke and benzo(a)pyrene concentrations in the air might have had some bearing on the recent decline in death rates, it seems unlikely that exposures to any component of air pollution could account for the massive increase that occurred earlier. Certainly there is nothing in these trends to implicate exposure to diesel fumes in particular, for the rise in death rates began before these could have had any impact and the decline started when concentrations would have been increasing rather than decreasing.

It is of course clear from the many epidemiological studies that have now been done (e.g., Doll and Peto, 1976) that smoking is the dominant factor in the aetiology of lung cancer, and the trends displayed in Figure 1 can be accounted for largely by the switch from pipe to cigarette smoking amongmen earlier this century, with the downward turn in the curves reflecting effects of reductions in smoking in more recent years coupled with a change towards filter cigarettes with a lower tar yield.

A better impression of the possible effect of air pollution can be obtained by examining trends in urban and rural areas separately. Table 2 shows part of a large table that has been prepared, giving sex-age specific death rates for lung cancer in Greater London and in rural areas of the country. The main feature is that while death rates in London have always been higher than those in rural areas, the gap between them is closing gradually. These trends also may be dominated by changes in smoking habits as the latter become

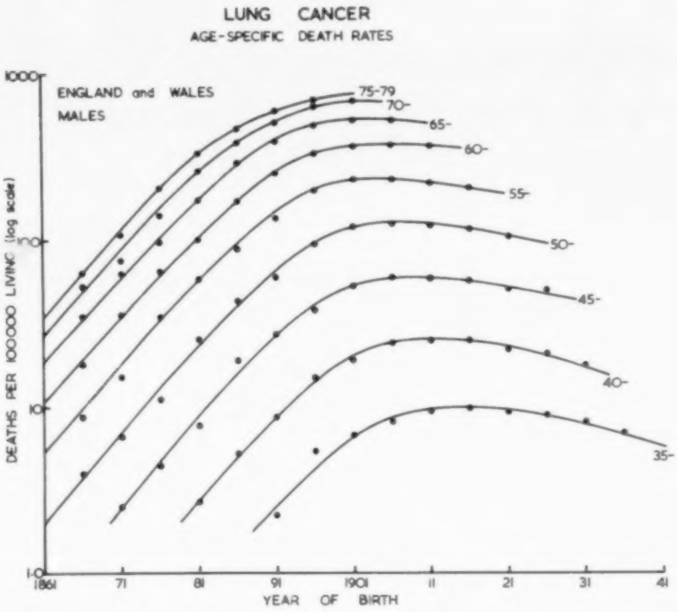

Figure 1. Trends in age-specific death rates for lung cancer (males) in England and Wales, arranged in relation to year of birth.

Table 1

CONCENTRATIONS OF BENZO(A)PYRENE IN AIR AT SITES IN CENTRAL LONDON, 1949-73, BASED ON 24 HOUR SAMPLES AGGREGATED FOR YEARLY PERIODS

Period	Sampling site	Benzo(a)pyrene g/1000 m^3
1949-51	County Hall	46
1953-56	St. Bartholomew's Hospital	17
1957-64	County Hall	14
1972-73	St. Bartholomew's Hospital Medical College	4

Table 2

LUNG CANCER: AGE SPECIFIC DEATH RATES, MALES AGED 60-64, GREATER LONDON AND RURAL DISTRICTS OF ENGLAND AND WALES

		Deaths per 100,000	
Year of Death	Year of Birth	Greater London	Rural Districts
1951-55	1891	350	170
1956-60	1896	420	240
1961-65	1901	450	270
1966-70	1906	405	295
1973	1911	370	290

These rates are approximate, being interpolated from tabulations in broader age-ranges. Figures for the single year 1973 are given as an indication of those in the most recent quinquennium, and these are subject to revision.

more uniform between town and country, but the downward trend in London could be related to the decline in pollution there. Clearly there is no indication from these trends that pollution from diesel engines, that is liable to have increased rather than decreasedin recent decades, has had any perceptible effect on lung cancer mortality in the general population.

To consider possible effects of pollution arising specifically from diesel engines it is necessary to turn to occupational groups with enhanced exposures. Men who work in garages when diesel-engined bases are parked and serviced present one opportunity, and to follow that up, Raffle (1957) examined lung cancer incidence among several categories of London Transport employees during the five-year period 1950-54. It was shown then that the incidence among the diesel bus garage workers was no higher than that expected among the general population. The duration of exposure of the men involved to diesel fumes might however have been quite short up to that time, since diesel buses were only introduced in the 1930's, gradually replacing petrol-engined vehicles until, in the 1950's they finally replaced trams and trolley-buses also. It is however now possible to consider the situation over a much longer period, since records of lung cancer cases arising during service among men in five categories (bus drivers, conductors, engineers in bus garages, engineers in the central works, and motormen or guards on the Underground) have been maintained continuously since 1950. Some preliminary results from this simple incidence-rate study for the 25 year period up to 1974 are presented below, and the analysis will be completed when data up to 1979 have been assembled, to make 30 years in all.

The study is limited to men in the age-range 45-64, and the numbers employed year by year in the five job categories considered are shown in Figure 2. In the early years there were some 20,000 men in all, but reductions in the number of vehicles in service, and changes in operating procedures, have led to a gradual reduction in the numbers employed in connection with buses. The change in numers has been accompanied by a changed in age-structure, and it has been essential therefore to define the population at risk in narrow (5 year) age ranges ready for the subsequent calculation of expected numbers of lung cancer cases.

The lung cancer cases comprised those recorded on death while still a member of staff within one of the designated job categories, plus transfers to alternative work within London Transport or ill-health retirement following diagnosis of the disease. In this way all cases arising during

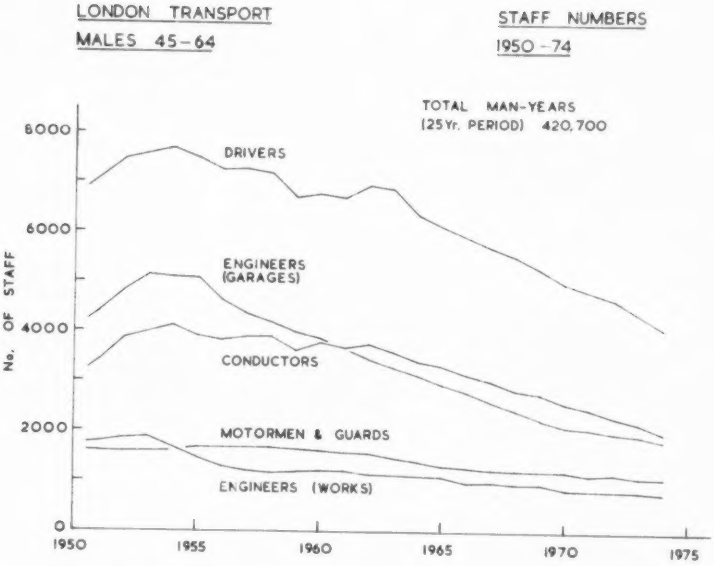

Figure 2. Numbers of staff in five job categories in London Transport, 1950-74.

service could be related strictly to the population at risk in the appropriate category. Ascertainment was believed to be complete, within the limitations of normal diagnosisand death certification, but some care was needed to ensure that cases involving transfer to alternative work were not counted a second time on death.

Ideally, incidence rates in the general population built up in a similar manner from first diagnosis or death records would be required for comparison purposes. Such data are available from cancer registries for recent years, but in a study extending back to 1950 it was considered more satisfactory to use death records only. In a disease with such a poor survival rate as lung cancer, the difference between these two bases is not substantial, and the question of any bias introduced by limiting general population comparison data to death records only is discussed further below.

Detailed tabulations of death rates in the general population had been prepared as part of a wider study of trends in lung cancer mortality in urban and ruraly areas of England and Wales, illustrated in Figure 1 and Table 2 above. Most of the Lond Transport staff lived within the Greater London area, and expected deaths were therefore calculated by applying Greater London rates to the population at risk within each job category. The data were assembled in 5 year periods and 5 year age ranges, finally aggregating results to obtain the total expected deaths in the age range 45-64 for the period 1950-74.

There were in all 667 cases of lung cancer reported over the 25 year period. The majority (81%) were notified on death, and the proportion of other cases (transfers to alternative work, or ill-health retirement) was greatest for bus drivers, possibly as a reflection of the greater need to remove them from their existing job on initial diagnosis of the disease. The numbers of cases occurring within each of the job categories are shown in Table 3, along with the numbers that would be expected on the basis of Greater London death rates. In each category the observed numbers are below those expected, and this may in part be a reflection of the "healthy worker" effect that has been seen in other studies of occupational groups subject to some degree of medical screening on entering employment. This is not however likely to be an important factor in the case of a disease such as lung cancer, that does not manifest itself at an early stage.

While the highest mortality ratio is that among bus garage workers, it does not differ significantly from that among motormen and guards on the Underground, who do not have

Table 3

LUNG CANCER CASES AMONG LONDON TRANSPORT STAFF IN RELATION TO THE NUMBER EXPECTED ON THE BASIS OF GREATER LONDON DEATH-RATES

1950-74, MALES AGED 45-64 ONLY

Job Category	Man-Years at Risk	Expected Deaths	Observed Cases	Mortality Ratio, %
Bus Drivers	175,909	346.8	259	75
Bus Conductors	93,095	174.5	130	75
Engineers, Garages	86,054	197.1	177	90
Engineers, Central Works	30,031	63.2	42	66
Motormen and Guards	35,610	67.7	59	87
Total	420,699	849.2	667	79

any special exposure to diesel fumes. The major problem in interpreting the differences in mortality ratios between the several job categories is however that the smoking habits of the men are not known. The study is based only on existing staff records, and there has not been any opportunity to collect personal information from the men.

The results can perhaps bets be put in perspective by linking them with the data on lung cancer and smoking published recently by the Office of Population Censuses and Surveys (1978). It has been shown that in England and Wales as a whole, lung cancer death rates within broad occupational categories are correlated with the proportion of (current) smokers in those categories. The findings are reproduced in Figure 3, identifying the point representing transport workers in the general population, and then superimposing the data from the present London Transport study. For this purpose the lung cancer cases have been expressed as percentages of the numbers expected i nthe population of England and Wales rather than Greater London, to allow direct comparison with the national data in terms of SMR's (Standardized Mortality Ratios). Clearly there are limitations to this approach, including the different time spans involved in the London Transport and the national data, but sine there has been no substantial change through the years in the ratio of observed to expected cases in the London Transport series, then the comparisons should be valid. The conclusion to be drawn is that the London Transport data would be consistent with the national figures if the proportion of smokers among garage workesr and the motormen and guards was similar to that in the general population, and below average in the other groups. None of the London Transport lung cancer rates is as high as that among transport workers in general. The inclusion of a small proportion of cases at diagnosis rather than at death in the London Transport series should not make any appreciable difference to the SMR's, although if some of those men lived on for several years (perhaps taking them beyond age 64) then the tendency would be to overestimate the London Transport lung cancer rates in relation to those of the general population.

A limited amount of environmental sampling has also been done in connection with this study. At the time of the original work by Raffle (1957), observations of smoke and the associated polycyclic hydrocarbons were made at two representative bus garages in London (Commins *et al*., 1957). The conclusion then was that while concentrations of smoke were substantially enhanced in the garages, the amounts of polycyclic hydrocarbons present were in general dominated by outside sources (principally coal fires), and it was necessary to select warm days in the summer to detect the effect of emissions from the buses.

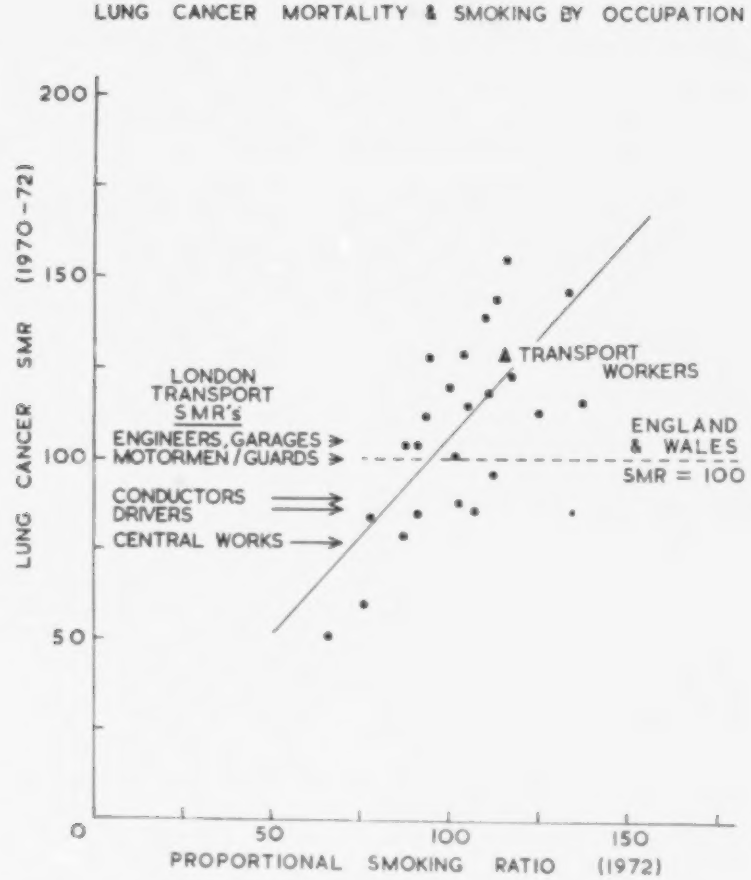

Figure 3. Lung cancer mortality and smoking by occupation, England and Wales, males 1970-72.

In view of the changes that have taken place during the past 20 years, both in terms of the control of emissions from coal fires and of the types of buses in services, further sampling has been done recently int he same two garages as before. Concentrations of smoke and polycyclic hydrocarbons have again been determined, and Figure 4 illustrates findings from just one section of the results, for benzo(a)-pyrene at Dalston Garage. This shows results for a single day, divided up into periods of several hours at a time, corresponding with different phases of activity in the garage. The lower part of the diagram relates to the recent measurements, and corresponding data for 1957 are included above for comparison. It can be seen that the background concentrations of benzo(a)pyrene in the outside air are now very much smaller than they were, and the contributions from the buses (represented by differences between the unshaded and shaded columns) are also mainly smaller. The present concentrations are clearly very small in comparison with those that used to exist in the general air of London (particularly during the winter months) some 20 or more years ago, and the indications are that the overall exposure of garage workers to benzo(a)pyrene during their working lives would not differ much from those of the general population. There is no doubt though that their exposure to smoke would be greater than that of the general population, since concentrations have at all times been higher in the garages than outside. It may be that there are other constitutents of the smoke with potential carcinogenic activity, but the epidemiological data reported above o not indicate any increased risk of lung cancer that may be attributed in any way to the diesel fumes.

Despite these negative findings, arrangements are in hand to continue and extend the present investigation, and for some groups of workers it will be possible to carry out a cohort study, in which the men will be followed up to death irrespective of whether they remain in employment with London Transport.

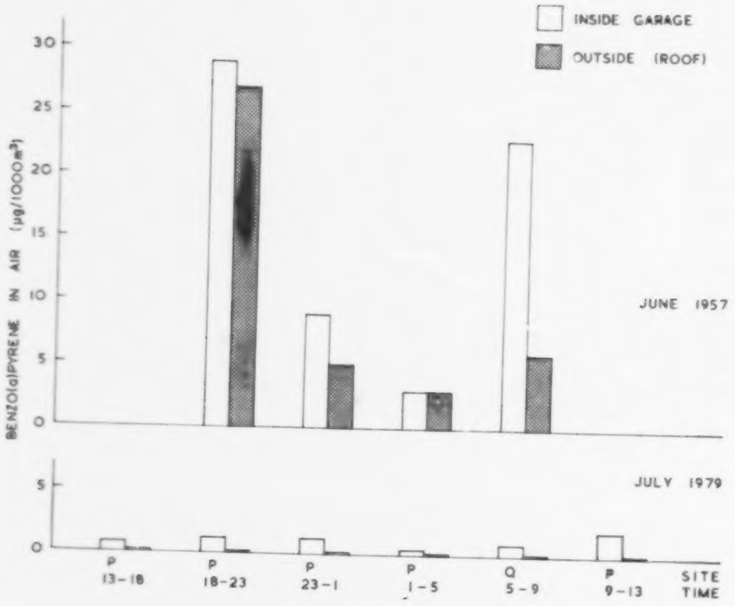

Figure 4. Concentration of benzo(a)pyrene in Dalston garage: 1979 compared with 1957. Site P was at the main entrance to the garage and Site Q at one end, close to parked buses.

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General Discussion

L. JOHNSON: At the beginning of your presentation you showed a dramatic decrease in black smoke in London and attributed the remainder to diesels. Do you have any idea of the proportion contributed by light-duty, heavy-duty, and stationary diesels?

R. Waller: No, much of the pollution in the early years could not have been contributed by the diesel, but it is only in the most recent times, since about the early '60's, that diesel pollution has become significant.

A. Kolber: I noticed an absence of morbidity data in your studies. I have tried to do some work of a similar nature and also find a great lack of morbidity information in this country. I thought that morbidity information was available in England though.

R. Waller: It is, yes. The study I presented, however, was just related to mortality. It is not quite clear whether we can really rely on this paper. We have social security records too and these have been used. One has seen differences in occupational groups in sickness, but it is by no means certain that these really reflect true health effects.

J. HANCOCK: Were these mortality causes all from death certificates?

R. WALLER: All those presented here were obtained from other sources. The death certificates are slightly better than the rest. Some do not reflect the death certificate but those carrying authorization have their own card and when a diagnosis such as mine is made, or anything that is related to it, they would show it. I would say that the diagnoses are as good as, and possibly better than, the general population at large.

F. SPEIZER: I appreciate why you are continuing your study for another five years. There might also be other ways of analyzing the data. This is a question that epidemiologists run up against all the time, estimating what the appropriate expected numbers are for a population such as this. It is not totally clear to me that the general mortality for the greater London area is the appropriate expected value. If you try to use one of the other working groups to estimate the expected values, it looks to me as though you might get relative risks for the engineers that might approach 1.5, 1.6.

R. WALLER: We have taken the whole and tried to look at the data of that population and then looked at respective values for all of the engineers and they are higher. You will perhaps note that another factor is time and the underground workers. As you can see, these are the least likely to be exposed to pollution, and looking at the trends over the years, we also tried to decide whether any of the groups have been increasing more rapidly than the general population or decreasing more rapidly. The underground men seemed to have come up slightly, more rapidly, than any of the others. It brings in all kinds of other problems when you ask what are the comparison groups. There is another problem and that is the ethnic origins. They actually went out in the West Indies to recruit workers and brought them back. This I think will also pull their weight down because these other ethnic groups have lower rates of lung cancer. We will then have to consider how different from the general population these are.

F. SPEIZER: That raises a further question in regard to when some of these people started smoking and how smoking affects some of these groups as well.

R. WALLER: I didn't go into detail. In fact, we have not done sufficient tracer studies. I think the ethnic groups have started smoking later than the others, and I think some groups increased more rapidly because they have caught up.

D. HOFFMANN: I understood it is fairly difficult, in retrospect, to get the smoking data on these people. Isn't it possible that there are some nonsmokers in this country? When I collected data there was never a time that more than 60 percent of the male population were cigarette smokers. When you have your 600 lung cancer cases, and I guess in England when you didn't smoke you were somewhat unusual, isn't there a way in retrospect to see how many nonsmokers were diseased?

R. WALLER: In the cases, or in the whole population?

D. HOFFMANN: No, in your 600 cases?

R. WALLER: Yes, it is possible. We have gone back to the records. There are some notes in the history, so we may make some point on this.

HUMAN DATA ASSOCIATED WITH DIESEL EXHAUST

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ABSTRACT

A review of the literature concerning data relative to human exposures from diesel engines is presented. Epidemiologic evaluations among workers exposed to diesel exhaust are discussed. Morbidity and mortality data are critically reviewed. The outline of a mortality and morbidity study to be completed in 1981 among construction workers exposed to diesel exhaust is given.

INTRODUCTION

Government and labor sources have speculated that diesel exhaust exposures may have adverse health consequences. It is widely recognized that the number of components within diesel exhaust are extremely numerous. The diverse emission stream of diesel exhaust is similar in complexity to that which we experience every day in the form of gasoline exhaust and urban smog.

The diversity of substances within the exhaust stream from diesel engines, in combination with the lack of comprehensive animal and human data on the subject, make estimates of health effects extremely difficult. Yet, circumstances dictate that at certain periods evaluations and decisions become necessary. At this point in time, the following discussion will attempt to place a perspective on the health implications of diesel exhaust exposures.

HUMAN STUDIES

Although a number of studies have attempted to evaluate the health effects of diesel exhaust on animals and humans, none of these efforts have looked at ambient exposure levels of diesel exhaust as would be experienced by the general population. The available human response data from diesel exhaust exposures have either been collected from occupational groups or from volunteers exposed to simulated occupational exposures.

Those constituents found in diesel exhaust that are of primary health concern are oxides of nitrogen, particulates and sulphur oxides. Since control of sulphur oxide emissions is possible by use of sulfur-free fuels, it might be considered that the agents of primary concern are oxides of nitrogen and particulate matter. For the sake of simplicity, particulate matter can be categorized in terms of carcinogenic and non-carcinogenic response. A great deal of information has been focused on benzo(a)purene (BaP) as an occupational and environmental carcinogen.

While it must be recognized that use of BaP as an index for all polynuclear aromatic hydrocarbons (PNA) leaves much to be desired, it remains as one of the better indexes for PNAs.

Earlier reports (1, 2) have estimated that PNA levels in many occupational settings, as indexed by BaP, are roughly similar to those levels found in ambient urban environments. If BaP can be used as an index of the carcinogenic hazard of diesel exhaust, then the following discussion of occupational environments has relevance to ambient diesel exhaust exposures.

The following will discuss health data from epidemiologic studies among diesel bus workers, diesel railroad workers, underground coal miners exposed to diesel exhaust and underground non-coal miners working with diesel equipment.

The general categories for human studies involving exposures to diesel exhaust emissions can be conveniently organized into two distinct categories.

The first category would involve those studies that look at lung-function testing and evaluate short-term symptoms. Data from medical questionnaires is used to determine subjective symptoms of respiratory health status. The second category involves mortality studies looking at death certificates among occupational cohorts. These studies are more useful in terms of evaluating long-term diseases, including cancer.

SYMPTOMATOLOGY AND LUNG FUNCTION TESTS

In 1964, Battigelli et al. (3) reported on cohorts of railroad workers with respect to occupational exposure to diesel exhaust emissions. In this study, there were 210 workers in the exposed cohort having an average of 9.6 years of exposure with a 154 worker control group. Results failed to show a significant difference in pulmonary function performances between the two groups. There were observed differences which were significant with respect to respiratory complaints associated with smoking habits.

In one of the only investigations of its type, Battigelli (4) exposed volunteers to diesel exhaust emissions for short periods of time and recorded pulmonary resistance. The exposure concentrations of the controlled diesel exhaust exposures were comparable to realistic values found in railroad shops. The results found no significant difference in pulmonary resistance among the exposed and non-exposed volunteers.

A study by El Batawi and Noweir (5) in 1966 reported results of a questionnaire survey of Egyptian workers in diesel bus garages. The reported findings were based on subjective complaints in the exposed population. Larger than expected prevalence rates of upper respiratory tract disease, chronic bronchitis, asthma, peptic ulcers, gastritis and high blood pressure were observed. The investigators reported that many of the workers were heavy smokers. It was suggested that the observed symptoms could be easily attributed to excessive tobacco consumption habits.

Jorgensen and Svensson (6) reported results of an investigation of pulmonary function by categories of smoking, age, and surface work versus underground employment in Swedish iron ore mines. Basic findings were that smoking and working underground were synergistic with respect to frequency of chronic productive bronchitis. Symptoms appeared early among underground miners who smoked; however, a falling off of symptom frequency was observed with increasing length of underground employment. Non-smokers underground exhibited a frequency of chronic bronchitis that was similar to smokers employed on the surface. The diminishing frequency of respiratory tract infections in these underground miners is inconsistent with a decrement in immunologic competence. While the results of the study demonstrated some age-related differences with respect to pulmonary changes, the authors stated that no significant differences were observed that could be related to employment above or below ground.

In 1969, Yamazaki et al. (7) reported on a study evaluating the effects of diesel exhaust gases on railway workers. Lung function equipment included a spirometer and a peak flowmeter. A total of 475 workers were observed. The control group consisted of 67 individuals not receiving diesel exhaust exposures during their work routines. Questionnaires were used that included data on the type of work site, age, job designation, air pollution, residence, allergic factors, heart disease history, subjective respiratory symptoms, tobacco habits, height and weight. No reductions of pulmonary function were found in excess of 10 percent for the five respiratory function variables tested. Inspection and repair sheds showed results that were greater in magnitude in comparison to workers assigned to tunnel areas. Work sites had greater observed effects on pulmonary function results than smoking. Data analyses utilized multi-variate statistical techniques relying on quantification theory. It was concluded that no significant effects were observed as demonstrated by the failure to obtain a greater than 10 percent reduction of pulmonary function.

In 1978, Attfield (8) reported on a respiratory health survey taken in 21 metal and non-metal mines. This study used a combination of lung function tests and questionnaires based on those of the Medical Research Council of Great Britain. A total of 4,924 men were evaluated. The purpose of this effort was to update a former study in silica mines performed by the Public Health Service in 1958 through 1961 (9) and correlate health effects to measures of diesel use and silica exposure levels. The analysis evaluated only white male miners. Several parameters of exposure were evaluated. The variables included:

1. number of diesel machines in use;
2. years of underground exposure; and
3. exposure to NO_2 and aldehydes.

Symptoms of persistent cough, persistent phlegm and shortness of breath did not demonstrate any association between the amount of diesel units in use and adverse health effects.

No consistent associations between three indices of lung function (FVC, FEV, and FEF_{50}) and diesel use were found. The general picture presented by the analysis is one of conflicting results. There was an observed increase in lung function with increasing length of diesel exposures.

While it is plausible that diesel exhaust was not harmful in these mines, it was reported that other possible reasons could account for these negative findings. These negative findings may have occurred for a number of reasons including the observed wide variation in the length of underground exposure, the potential for the indices of pulmonary function to have been inappropriate, and the possibility that the differences between high and low exposure categories were not significant.

Gamble, et al. (10) reported on a study that evaluated workers at five salt mines. Two of these mines used large amounts of diesel equipment while two mines used lesser amounts. One mine did not utilize diesel equipment. The study was designed to determine if diesel particulates plus nitrogen dioxide exposures were associated with pulmonary function changes over a work shift. A total of

246 miners were tested. Maximum forced expiratory volume in one second (FEV) and forced vital capacity (FVC) values were used to obtain peak flow and flows at 25%, 50% and 75% (FEF_{25}, FEF_{50}, FEF_{75}) of FVC. The analysis of these data demonstrated a decrement in pulmonary function associated with nitrogen dioxide exposures. No decrement in pulmonary function was observed with respect to diesel particulates.

In 1979, Reger and Hancock (11) reported on a study that evaluated miners working in coal mines using diesel-powered machinery. This study, which used matched pair analysis, evaluated pulmonary function tests and questionnaires. Data from miners exposed to diesel exhaust were matched to miners working in coal mines not using diesel machinery. The data for the miners in the five coal mines using diesel equipment were collected in 1978. The data for those miners working in coal mines not using diesel machinery were derived from the (1973-1974) second round of the National Coalworkers Pneumoconiosis Study. The response rate for the exposed group was 92 percent. The response rate was not reported for the control group. Selection from the control group to constitute the "matched pair" for each exposed miner was done to correlate the variables of geographic area, smoking status, weight, height and years underground. In all, 722 pairs were evaluated. Incidence of cough and phlegm symptoms were greater for the miners exposed to diesel exhaust than their non-exposed counterparts. The miners exposed to diesel exhaust, however, had significantly fewer complaints of moderate to severe dyspnea and wheezing compared to their non-exposed counterparts. Results of pulmonary function tests were mixed.

The mean difference in pulmonary function variables including FEV, FVC, FEF_{50} and FEF_{75} indicate on the average that the diesel-exposed group had statistically lower pulmonary function than controls. Conversely, the diesel-exposed miners had improved pulmonary function that was statistically significant (unpublished report - 12) in terms of peak flow in comparison to controls.

Some interesting associations were observed when the pulmonary function data was segregated by individual mines. Among those miners evaluated in Mine 4, improved lung function differences for FVC and FEV were observed in comparison to their matched controls. On the average, the men of Mine 4 were reported to have longer exposures to diesel emissions in comparison to the other diesel mines evaluated. Differences in pulmonary function for Mine 3 were markedly greater than for any of the other mines. Of the five pulmonary function variables tested, decrements in pulmonary function among the miners exposed to diesel exhaust were found to be statistically significant for three variables in Mines 1 and 3, and a corresponding decrement for two variables was observed in Mine 2. Of the five pulmonary function variables evaluated for the matched pair differences in Mines 4 and 5, one was found to be statistically significant (Peak Flow, Mine 5) in terms of improved pulmonary function (unpublished report - 13) among the diesel-exposed mines and another variable (FEF_{75} - Mine 5) was statistically significant for a decrement in pulmonary function. The eight remaining differences among the matched pairs in Mines 4 and 5 were statistically insignificant. The lung function and symptomatology data lack sufficient consistency to allow any conclusion regarding the health effects of diesel exhaust.

MORTALITY STUDIES

In 1957, Kaplan reported on a mortality study on railroad workers for the years 1953-58 inclusive (14). The workers were divided into separate groups: operating personnel exposed to exhausts from diesel and steam engines (e.g., trainmen) and non-exposed workers (e.g., office personnel). The study comprised 235,110 person-years. A total 6,506 deaths in the study population were reported; only 154 were attributed to lung cancer, although 192 would have been expected on the basis of age-specific rates. There was no increase in lung cancer mortality in any of the groups studied. Lung cancer among London transport workers servicing bus equipment was reported by Raffle in 1956 (15). This study found no excess of lung cancer "among any group of London transport staff such as would have been expected if diesel exhaust were a serious contributory factor in producing an excess of lung cancer in urban dwellers".

In England a recent follow-up study has been reported through the auspices of EPA which accounts for 25 years of exposures by diesel transport workers in London bus garages (16). The lung cancer rate for the garage workers was not statistically different from that of any other group of employees, such as underground train drivers and guards. Additionally, the lung cancer rate was less than that found among the general population.

An epidemiologic investigation performed by NIOSH evaluating the mortality experience of potash miners and millers was authored by Waxweiler, Wagoner and Archer in 1973 (17). Although the study was principally concerned with causes of mortality (especially lung cancer) related to the question of individual constitutional predisposition, two of the eight mines investigated had long histories of diesel engine usage for underground transportation purposes. One mine had used diesels for approximately 24 years and the other for approximately 16 years at the time of the study.

The investigators subdivided the underground cohort according to which men had worked in either of the two "dieselized" mines and when. On the basis of the mortality experience of the total cohort of underground miners, 46.7 deaths would have been expected in the dieselized population of 6,733 person-years when, in actuality, only 31 deaths occurred. This NIOSH study concluded that " ... no major cause of death exceeded expectations among men who had worked in the diesel-using mines." In addition, the study concluded:

> "... No causes of death were significantly different between miners who worked in dieselized mines and those who worked in other mines. It may be noteworthy that the 'other respiratory disease' category, which was high among underground workers, was not different between diesel and non-diesel workers."

The investigators stated that this latter finding may be related to the "insufficient elapsed time since the start of diesel usage for chronic or long latent-period diseases, such as emphysema or lung cancer, to be manifested as excess deaths in the relatively small exposed group." However, it should be emphasized that diesels had been in use in the two mines for approximately 16 and 24 years, respectively. This study represents the only mortality investigation published in the scientific literature that was conducted in a dieselized mine in the U.S.

HEALTH RESEARCH STATUS

To date, results from health studies on diesel exhaust exposures have been inconclusive. The human health literature has not shown any evidence that exposures from diesel engines can be associated with adverse chronic health effects such as cancer or other long-term illnesses. Future studies involving diesel exhaust with longer exposure periods to account for the twenty-to-thirty year latency period expected for chronic diseases such as cancer would help alleviate some of the present uncertainties in the assessment of the health hazards from diesel exhaust emissions.

It appears that two types of epidemiologic studies are needed to provide an improved health hazard assessment of diesel exhaust exposures among humans. The first type of study would be a cross/sectional morbidity study among a population exposed to diesel exhaust. This form of survey could be based upon an examination of an occupational population. Properly devised sampling techniques evaluating the factors of age, race, geographical location, job location, and other variables effecting exposures would be necessary to assure both representativeness of the sample and delineation of distinct exposure categories. Morbidity studies have limitations in terms of extrapolating results to chronic illness. In the case of diesel exhaust, such studies would help evaluate the acute effects on respiratory and other organ systems effected or associated with diesel exhaust exposures.

Chronic health hazards including cancer endpoints can be evaluated by use of mortality data. Although a prospective epidemiologic approach would follow an exposed cohort for thirty years and could offer a more ideal study design, time constraints limit such approaches.

HEAVY EQUIPMENT OPERATORS

Currently in progress is an epidemiologic study designed to evaluate mortality trends and cancer incidence among an occupational cohort exposed to diesel exhaust. This study, which is scheduled for completion by 1982 (personal communication - 18), will evaluate the medical records from a Northern California union of heavy equipment operators. Mortality patterns in the selected cohort will be compared to those expected among other males of the same age in California or other appropriate areas within the United States. The hypothesis of the mortality study is, "Do men exposed to diesel exhaust exhibit different mortality patterns than similar workers without exposure to diesel exhaust?"

The cohort will be selected from those individuals having one year of work history between a period of January 1, 1958, through December 31, 1978. The eventual cohort is estimated to comprise some 25,000 to 40,000 individuals and will consist of up to 500,000 man years of working experience.

This cohort will also be evaluated in terms of cancer incidence. This procedure will utilize the California Tumor Registry (CTR), which contains the incidence of all tumors reported within five San Francisco Bay Area Counties contributing to the CTR. This phase of the study will use membership computer tapes and correlate the data with members residing in the five Bay Area Counties who were alive and had a working year of membership by the end of 1969. Individuals joining this union after 1969 will be included in the cohort following one year of their date of initiation. This cohort will be matched with those cancer patients in the five Bay Area Counties contained in the CTR on an annual basis beginning with 1969. It should be possible to identify each local union member with one year or more membership and evaluate the incidence of tumors. This cancer incidence study will have an advantage over the mortality study because it will pick up the risk of developing tumors as it includes those exposed workers with tumors that are living.

An important aspect of these efforts will be the environmental sampling component. The environmental sampling is designed to evaluate levels of diesel exhaust exposures that characterize the study populations in terms of work classifications. The sampling procedures will evaluate numerous job sites where construction workers are involved with heavy equipment using diesel-powered machinery.

Another feature of this epidemiologic study will enable a data collection network to form a worker health registry. This health registry can be updated and used for future prospective epidemiologic evaluations of working cohorts with longer exposure periods to diesel exhaust.

SUMMARY

The preceding has discussed the available human diesel health literature with respect to both short-term and chronic indicators of health. Studies looking at lung function data and respiratory complaints from questionnaires were evaluated. No conclusive evidence suggesting an association between diesel exhaust exposures and adverse health effects have been established. Mortality studies evaluating cancer incidence among occupational cohorts have also failed to associate diesel exhaust exposures with long-term diseases, including cancer.

More studies involving humans evaluating short-term and long-term health effects are urgently needed. A review of a study among construction workers exposed to emissions from heavy equipment powered by diesel engines was discussed. While the entire question of human health effects from diesel exhaust cannot be settled on the basis of this particular study, the large cohort should help clarify the question of whether or not diesel exhaust exposures adversely affect workers in terms of long-term illness.

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SPEAKER: On pack years of cigarette consumption, they are matched qualitatively exactly as to the smoking status. We thought it was unnecessary on the basis of pack years because as you know they were also matched fairly well on age. Later on we have done as you have done with the data also looking at pack years and as you might expect, they are roughly equivalent overall even though the matched pairs vary between them in pack years. Secondly, on the study of acute effects the changes in lung function over a work shift indicated that the controls we used were older than the exposed people. This was all we had at the time and this did worry us to some extent. However, we found that the age of a person seemed to have almost no importance whatsoever relative to the pulmonary performance of the individual over an eight hour work shift. Hence, I think the age effect in terms of an eight-hour shift change is somewhat cancelled out. Smoking certainly had an effect. No one denies for a moment that aging has an effect on pulmonary functions. Certainly it does.

D. LACHTMAN: That is a good point. I had thought that to be true from the data we got in the Hatfield Study. It is a fairly large study and I think it was in that study that we actually found some increasing results, with a decreasing time of the people - that is easy to explain. In terms of people smoking, you are right. You matched qualitatively very well, so we played with your first data batch.

S. **KAPLAN**: On the Environmental Health Associate study on cancer incidence, do you have any information as to what proportion of your teamsters migrate outside the five-county bay areas that is covered by the cancer registry? I think this is something that if it is going to be carried out will have to be addressed in your analysis.

D. LACHTMAN: That can be looked at. In fact, the numbers that must be followed include some of the teamsters who work in that union but don't live in the bay area. These will have to be eliminated and it is going to decrease the numbers considerably. I think there is some analysis being done in that regard but I don't think it is complete.

S. KAPLAN: I am also talking about people who lived there at the time thay qualified and then subsequently moved away, who will not be picked up.

A RETROSPECTIVE COHORT STUDY OF DIESEL EXHAUST EXPOSURE IN RAILROAD WORKERS: STUDY DESIGN AND METHODOLOGIC ISSUES*

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ABSTRACT

Despite the experimental evidence for a carcinogenic effect of diesel exhaust, only a few epidemiologic studies have evaluated this question. All of the existing studies have major study design or methodologic weaknesses that may account for the absence of a consistently observed effect. Some of these issues which are discussed in this paper include source of subjects, required sample size, cohort selection and confounding. Study design issues are related to a proposed epidemiologic study to evaluate the possible carcinogenic effect of diesel exhaust in U.S. railroad workers. Data for the study come from the U.S. Railroad Retirement Board. Components of the study include: 1) a retrospective cohort analysis of approximately 80,000 male railroad workers, 2) a case-control study of 300 incident lung cancer cases and matched controls in railroad workers, and 3) actual environmental monitoring of worker exposure to diesel exhaust.

These approaches will allow for quantitative assessment of both level and duration of diesel exhaust exposure and consideration of the major confounding factor (cigarette smoking), thus minimizing the major drawbacks of all previous studies.

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An epidemiologic study of diesel exhaust as a potential carcinogen presents many unique methodologic problems in addition to the more general difficulties of studying a possible occupational or environmental carcinogen. I would like to discuss some of these study design problems and relate them to previous epidemiologic investigations of diesel exhaust effects. Finally, I will describe a study we are undertaking of this question on U.S. railroad workers and relate some of these issues to that study.

Study Design

A few large scale cross-sectional cancer surveys have recorded occupation data and determined cancer rates, particularly for respiratory cancers, in occupations with potential diesel exhaust exposure. These studies have found increased Standardized Mortality Ratios (SMR's)† in diverse occupations with possible diesel exposure including construction workers, railroad brake and switchmen, and truck drivers (2,3). Such large descriptive surveys can only be considered hypothesis generating since positive findings may be due to other exposures, confounders or chance alone. In addition, occupation data are limited and potentially biased, usually being based on only the last known occupation. A final weakness in these studies is that quantitative and qualitative exposure to diesel exhaust or any other substance is only inferred.

Analytical or hypothesis testing studies are basically of two types, case-control and cohort. A case-control approach has been used at Roswell Park Memorial Institute to look for trends in cancer incidence by occupation (4). They compare occupation in patients with cancer to the occupation of patients who are found not to have cancer. This approach can control for confounding factors such as smoking habits, but results relating to diesel exposure have been limited by small numbers of cases in individual occupations, crude occupational histories and no data on actual exposures. In these studies no significantly increased relative risks (RR's) in lung cancer for railroad engineers or firemen or for truck or bus drivers were found (4,5).

Case-control and cohort studies have also had reduced statistical power in studying possible causal effects of diesel exhaust and cancer because of the absence of a "marker" tumor, as is the case for mesothelioma from asbestos or angiosarcoma from vinyl chloride exposure. While such a "marker" tumor does not exist for diesel exposure, the use of more specific histologic classification of tumors in epidemiologic studies may increase the sensitivity and specificity of these investigations. An excess of oat cell carcinomas of the lung has been noted in populations exposed to known occupational carcinogens including asbestos, chromium, uranium and chloromethyl methyl ether (6-8). A case-control study of oat cell cancers of the lung from the Massachusetts Tumor Registry found an excess of transportation equipment operatives compared to controls with central nervous system tumors (9). While this result is not specific for diesel exhaust, it suggests that pathologic assessment may be a potential tool for greater power in future case-control and cohort studies of diesel exhaust exposed populations.

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† The SMR is the number of deaths, either total or cause-specific, in a given occupational group expressed as a percentage of the number of deaths that would have been expected in that occupational group if the age-and-sex specific rates in the general population had obtained (1).

Most of the existing studies have used a cohort design and the remainder of this paper will focus on that epidemiologic approach.

Source of Subjects

A major weakness of existing studies that relate to diesel exhaust exposure is the absence of exposure data for different occupations. Extensive use of diesel engines is found in heavy duty trucks, bus and construction, railroad and maintenance vehicles and in some types of mining. Within these industries maintenance and repair personnel may have higher exposures than drivers of diesel vehicles (10), but there are scant data on actual exposure levels. For example, exposures of truck drivers or railroad engineers must be measured before these occupational categories are appropriately considered exposed to diesel exhaust. A Finnish study of railroad conditions found particulate levels over five times as high in diesel roundhouses as in diesel locomotive cabs (10). This type of information will increase the sensitivity of future epidemiologic studies but has not been utilized in the design of studies done up until this time.

A related issue is what component of diesel exhaust should be considered in evaluating exposure dose. Experimental evidence suggests that the particulate fractions, which contain the polycyclic aromatic hydrocarbons (PAH), are the principle carcinogenic components of diesel exhaust (11-14). However, particulate fractions may vary in their PAH content, and other exhaust gases such as NO_2 and SO_2 may modify the carcinogen effect (15,16). Data are only beginning to be generated on comparability of diesel exhaust from different sources.

Number of Subjects

Considering only cancer of the respiratory tract one can make estimates of the number of cases required to see an effect due to diesel exhaust exposure. These estimates use an approximation of equal potency of equal amounts of benzo(a)pyrene (B(a)P) in products of coal combustion and in diesel exhaust. Using estimates developed in the British Gas Works studies, the expected relative risk may be in the range of 1.5 to 2.5 (17). Existing measurements of diesel exhaust exposure suggest levels will not exceed the mean concentration of $3\mu g/m^3$ B(a)P to which the gas workers were exposed, however, further environmental monitoring studies are necessary to determine actual exposures (10,18). There are no data to suggest that exposures will equal those of coke-oven topside workers, where RR's as high as 10 for lung cancer were seen (19).

The rate of lung cancer for an unexposed population can be determined from cancer mortality statistics (20). It is then a straightforward calculation to determine the required cases of lung cancer to observe a statistically significant result for different RR's (21). This is done in Table 1 for a probability of lung cancer in an unexposed population of 0.0259*, one sided test.

TABLE 1

REQUIRED CASES OF LUNG CANCER IN A COHORT STUDY TO OBSERVE RELATIVE RISK

RR	N = Cases of Lung Cancer	
1.5	2313	4850
2.0	687	1440
3.0	165	346
5.0	80	168
	$\alpha=.05$ $\beta=0.2$	$\alpha=.01$ $\beta=0.1$

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*This probability was calculated for the white male cohort described in the study at the end of this paper. It is based on age and sex specific respiratory cancer rates for Connecticut, 1962-64 (20).

The existing studies relating to occupational diesel exhaust exposure and cancer are listed in Table 2 with the number of cancers observed.

TABLE 2

Author	Population	Number of Cases
Kaplan, 1959 (22)	Railroad Workers	154, lung cancer
Hueper, 1955 (23)	Railroad Workers	133, lung cancer
Raffle, 1957 (24)	London Transport Workers	96, lung cancer
Hannunkasi, 1978 (25)	Railroad Workers	47, all cancers
Luepker, 1978 (26)	Teamsters	34, lung cancers

Even the largest study, which observed 154 cases of lung cancer, may not have had enough statistical power to exclude a false-negative result (β type error).

Obviously, numerous factors may alter these size requirements. Errors in exposure classification or differences in rates of smoking between exposed and unexposed populations will increase the number of cases required. A more general carcinogenic effect or an effect on non-malignant causes of death (e.g. respiratory disease) may decrease the size of the required cohort.

Cohort Selection

One important factor in selecting a cohort is adequacy of the observation interval for the latency period of lung cancer. Studies of other populations exposed to PAH's suggest an incubation (latency) period of 10 to 25 years (27). Investigations of possible carcinogenesis from diesel exhaust must allow for a similar interval from the beginning of potential exposure to the end of the follow-up.

A related consideration is the time period of use of diesel engines. Diesel locomotives were first used in quantity by the railroads in the 1940's and only by the mid 1950's did they represent even the majority of locomotives in service (Fig 1). A similar sequence of use was seen in buses and trucks. Prior to use of diesel locomotive, most railroad engines were steam units. These engines potentially exposed the workers to the product of coal combustion and to asbestos from the thick asbestos insulation around the boilers. Exposure of subjects in a cohort of railroad workers to these engines represents a potentially significant confounder in diesel exhaust exposure studies.

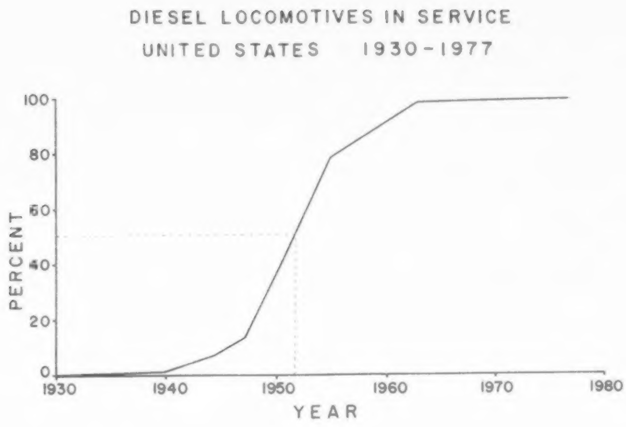

Figure 1. Diesel locomotives as a percent of all Class I locomotives in the United States. Docked line indicates 50% diesel locomotives in service, corresponding to the year 1952.

Thus, an optimal cohort for study must have adequate duration of exposure and interval of observation to allow for a 10-25 year latency period, but in the case of the railroads, workers cannot have had potential exposure much before the widespread use of diesels in the 1950's. Unfortunately, most of the studies of railroad workers and lung cancer deaths were done in cohorts exposed in the 1950's and earlier (Fig 2). They therefore cannot be considered to reflect effects of diesel exhaust.

Confounding

A final consideration in evaluating existing or planned epidemiologic studies of diesel exhaust exposure is the effect of potential confounders. A confounder is a factor related to the exposure being studied and also a risk indicator of the disease (28). The major potential confounder in diesel exhaust studies, particularly those assessing lung cancer or respiratory disease as an outcome, is cigarette smoking. Two considerations are important, 1) Is there a difference in smoking rates of the diesel exposed and the reference population, and 2) if a difference in rates exists, what is the direction and magnitude of any effect that might be seen.

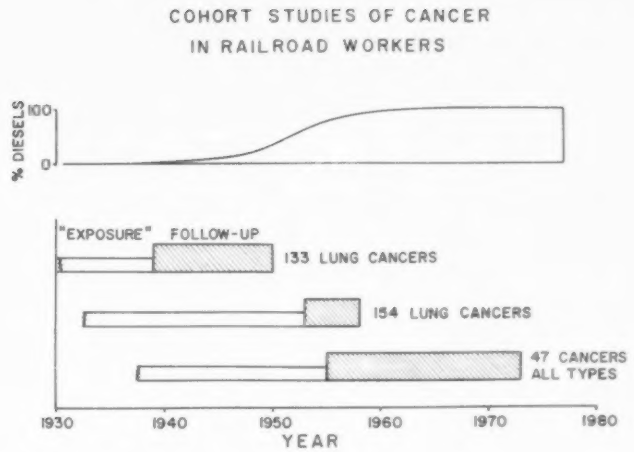

Figure 2. Three cohort studies of cancer in railroad workers. Percent diesel locomotives in service is plotted on the same time scale.

No cohort study on diesel exhaust effects contains direct information on smoking habits of the observed population. An estimate of present smoking in railroad workers can be made from a prospective study of cardiovascular diseases in U.S. Railroad employees that was begun in 1957-1959 (29). Comparing rates of current smokers in that study with data on U.S. males in 1955 obtained by the National Clearinghouse for Smoking and Health (30) indicates only small differences (Table 3). Differences in smoking rates of this magnitude can be shown to have only a weak confounding effect (31). The use of comparison populations within the industry being studied is another method to further reduce this possible confounding effect, particularly if one controls for covariates such as socio-economic status.

Proposed Study

This discussion has focused on reasons why the existing epidemiologic studies may have not been able to show an effect if one exists or may not have been specific for effects of diesel exhaust exposure. I would like now to briefly describe an epidemiologic study we are undertaking to evaluate this question.

The data base for the study comes from the files maintained by the U.S. Railroad Retirement Board (RRB) on all U.S.

Railroad workers. These computerized files contain demographic and job category information on all U.S. railroad employees in active service and on retirees with at least 10 years of service in the railroads. All male railroad workers between 50 and 64 years of age with 10 to 19 years of railroad service in 1964 will be potential subjects. From this pool a cohort of approximately 60,000 subjects will be selected (Fig 3). Selection will be based primarily on job category and specific railroad company. This will allow inclusion of workers in the highest diesel exhaust exposed job categories and from the companies with the longest history of diesel engine use. The cohort will also contain an appropriate control of workers in non-exposure job categories.

TABLE 3

Comparisons of Current
Smokers in Railroad Employees
and United States Population,
Male, Age 45-54

	Current Smokers (%)
U.S., 1955	56.0
Railroad Employees, 1953-1954	
Clerks	57.3
Switchmen	62.2
Executive	54.7

The cohort will be followed through 1979 and all deaths in active service and after retirement will be ascertained and coded for cause from death certificates. Based on U.S. male cancer mortality rates, one can estimate that 2,403 cases of lung cancer will occur. This should allow detection of a RR of at least 1.5 for lung cancer between exposed and non-exposed populations.

Another proposed component of the investigation is a case-control study of approximately 500 incident cases of lung cancer reported to the RRB for 1976-1978. Each case will be age and sex matched with a case of non-respiratory cancer and another case of non-malignant non-accidental cause of death. Next of kin of all cases will be contacted for questionnaire information on potential confounders such as cigarette smoking and residence (urban vs. rural). Hospital records will also be requested on lung cancer cases and histologic material reviewed by a consultant pathologist.

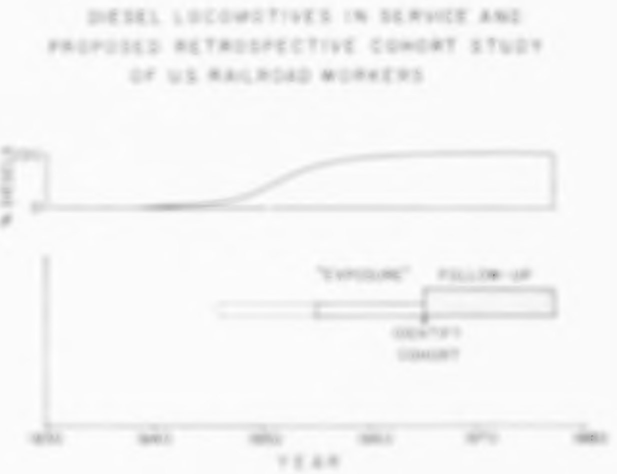

Figure 7. Proposed retrospective cohort study described in the text. Minimum potential exposure is 15 years. Percent diesel locomotives in service is plotted on the same time scale.

A third and very important part of the study will be actual environmental and personal monitoring of levels of diesel exhaust exposure for different job categories and work areas. This will be done in different parts of the country and over two seasons. This information will be integrated into the cohort selection and epidemiologic data analysis.

We hope this combined approach will provide useful evidence about the possible occupational carcinogenic effect of diesel exhaust and also relate to increased diesel exhaust in the general environment.

The retrospective cohort component will evaluate a unique population of workers that is both large enough and has an adequate duration of potential exposure and follow-up to detect a relative risk for lung cancer of 1.5. This component will be supplemented by case-control study of incident cases of lung cancer. By this method we will be able to directly control for potential confounding factors and to evaluate the occurrence of less specific causes of lung cancer. The use of environmental and personal exposure monitoring data in selecting the cohort for study and evaluating the data will greatly increase the efficiency and validity of the results.

As mentioned, this study is just beginning. We anticipate it will take 3 years to collect the appropriate information and complete the analyses. Thus, it will be an expensive study both in terms of time for people and money. However, since automobiles and light duty trucks are predicted to be 25% diesel by 1985, this study may be the last opportunity we have to measure the effect of one source of diesel exhaust before it becomes part of the general environmental pollution, in which case added complexity may make it impossible to select out the effects that might be present.

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CHARACTERIZATION OF DIESEL EXPOSURE GROUPS

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ABSTRACT

Current diesel epidemiological studies focus upon occupational exposures because public exposure to diesel emissions has until now been minimal. With the pronounced market penetration of diesel passenger automobiles predicted for the 1980s public exposure will be markedly increased. Care must be exercised for the utilization of occupational cohorts in order to derive the greatest benefit from their study. Each group will have its own characteristics of exposure which may vary across specific job classifications, worksites, and levels of activity to note but a few parameters of concern. Additionally all groups are subjected to accompany toxic agents which may act as confounding factors. Issues discussed in this paper include establishing criteria useful to contrast occupational groups to determine their suitability to test hypotheses on health effects and examining indicators of exposure and measurement methods.

EXPOSURE GROUPS

Current diesel epidemiological studies focus upon occupational exposures because public exposure to diesel emissions has until now been minimal. The groups that have been identified as possibly having above average exposures, include railroad workers, truck drivers, miners, heavy duty equipment workers, diesel mechanics, and trash collectors. Within each of the occupations, the exposure environment may vary according to job classification, worksite, and level of activity. Furthermore, each of these groups is exposed to other airborne agents, which may act as confounding factors.

In order to assess their applicability for utilization as an epidemiological cohort a great number of considerations need to be made including but not limited to:

- The statistical requirements of the study protocol
- The characteristics of the exposure including the period, level and the relationship across job or equipment classification
- Confounding factors such as other substances and working conditions.

Of course this merely touches on points needed for consideration of performing such studies such as population mobility, availability of records, etc.

In order to provide proper exposure data for an epidemiological study, two questions must be addressed:

1. What are reliable indicators of methods of measurement of exposure to diesel emissions in the working environment?
2. Do the levels of exposure differ sufficiently among occupational groups to enable the confirmation of hypothesis on health effects?

Diesel exposure measurements reported in the literature and obtained in field studies by SAI are summarized for comparison in Table 1. Although the wide variety of measurement techniques makes it difficult to compare values, it is evident that a measurement program must be carefully tailored to the exposure environment under study. Both personal and area monitoring for gases and particulates are necessary. Because of their importance in health effects, respirable particulates need to be collected and analyzed in such a manner as to separate and identify components of diesel origin. Techniques for these analyses will be described.

In attempts to determine whether there are significant differences in the extent to which individuals in different cohorts are exposed, several outcomes are possible:

- Exposures are essentially the same for all putative exposure groups
- Exposure differences are statistically significant but meaningless from a health standpoint
- Exposure differences are both statistically and medically significant
- Exposure differences become meaningful if exposure group categories are redefined.

A study design based upon a randomization or non-parametric test is expected to be useful in determining which of these outcomes applies.

Table 1 summarizes a group of studies which measured workplace concentrations of several diesel emission species. It is difficult to make meaningful comparisons with these data and/or to extract answers to either of the above posed questions. Difficulties are many and but to mention a few include:

- There are a wide variety of measured species, average times, conditions and techniques utilized.
- Little personal sampling has been done, rather area sampling is principally available.
- Significant changes in occupational equipment and conditions have occurred over the extended periods of interest.
- Determination of exposure across job categories, although necessary, is usually unavailable.
- Similarly the separation of exposure level from background level is essential but not usually conducted rigorously.

Looking at the table allows some gross intercomparison among occupations. Our first hand experience centers around buses and diesel tractors, earthmoving equipment, and truck repair sites. In all our studies attempts were made to determine and separate out the background and self contamination contributions. Several limited pieces of data not included in the table include other gases measured inside and outside of diesel tractors:

TABLE 1. SUMMARY OF DIESEL WORKPLACE AMBIENT CONCENTRATION STUDIES

Type of Workplace	Basis for Averaging	NO ppm	NO ppm2	CO ppm	Respirable Particulates mg/m3	References
Tunnel construction	U[a]	1.5-3.5	b	<10	0.5-1.5	Sutter, 1975[c]
Underground Coal Mine	U	5.3	0.5	6.5	ND[d]	Stewart, 1978
Underground Copper Mine	1-hr or 2-hr	11.4-25.6	0-2	8-16.3	1.4-6.5	Johnson et al., 1976c
Bus Garage	13 hrs	ND	ND	ND	0.30[e]-1.25[e]	Commins et al., 1957
Railroad Repair Shop	20 hrs	0.02-0.20[f]	0.01-0.06	1-15	0.09-0.26	Apol, 1973
Rail Theoretical Worst Case	8 hrs TWA	23.1	0.87	35.1	1.7	Sanders and Pecay, 1976
Underground Coal Mine	U	1-59	0.10-0.47	1.5-14	ND	Lawter and Kendall, 1977
Diesel Trucks	U	0.1-2	0.01-3	0-30	ND	Ziskind, 1977
Earthmoving Equipment	2 min	0.1-0.5	0.02-0.54	1-4	0.01-0.15	Kogozen, 1979
Truck Repair Shop	2 min	0.05-1.05	0.03-0.24	<1	0.09-0.21	Kogozen, 1979

[a] U = unknown.
[b] NO_2 concentrations were all less than 10 percent of corresponding values for NO.
[c] Cited in Stewart et al., 1978.
[d] ND = no data.
[e] Total particulate.
[f] NO = Reported "NO_x" - NO_2
[g] Estimated by assuming that NO/NO_2 = 10.

TABLE 2. EXPOSURE INDICATORS

	Supporting Features	Detracting Features
NO and NO_2	Reliably measured Sufficiently stable Interferences known Well differentiated from background Personal sampler available (NO_2)	Exposure well below TLV (NO) Relationship between $NO-NO_2$ varies markedly
CO	Stable Few interferences Personal sampler available	Not excessively emitted Poor differntiation from urban background Exposure well below TLV
SO_2	Personal sampler available	Less data available Fuel content varies Conversion to other species
Aldehydes	Known to be toxic Thought to be useful indicators of diesel exposure-over background levels	Field measurements present difficulties Cost competitive detection methods relatively insensitive
Particulates	Useful indicator (respirable) Personal sampler available Analysis permits differentiation from background	Area sampling is costly and useful only for longer period averages

Gas	Result of Summary
o Sulfur dioxide	o Approximately ambient levels
o Gaseous hydrocarbons (methane equivalent)	o Factor of 4 to 6 times ambient (20-27 ppm)
o Ozone	o Fraction of ambient

The review of the literature does not allow definitive guidelines to be formulated concerning the most useful exposure indicators. Summarizing the supporting and detracting features of the compounds usually measured we have the results in Table 2.

Special mention should be made concerning the role of particulate measurements. Clearly they rate a high priority in supporting an epidemiological study by quantifying exposure to potentially toxic emissions. In designing a recommended characterization of the particulate environment we have included the following:

- o Total and respirable (<2.5 μm) particulate fractions of both area concentration (upwind and downwind) and personal exposure should be taken
- o Diesel emission measurements - analyses for organic and elemental carbon
- o Upwind dust - analyses to determine elemental composition
- o Relative proportions of total mass, organic and elemental carbon, and carbonates should characterize diesel contribution and allow differentiation. The interrelationship between two or more elements such as aluminum and calcium could provide additional verification.

SAMPLING APPROACH

In order to evaluate the relationship of exposure within the occupational group being considered, i.e., the second question posed, comments are needed concerning structuring a sampling approach while considering the problem of large numbers of job classifications and/or equipment types being used. Thus one wishes to know how meaningful such divisions are and determine how to stratify our cohort into exposure groups not solely based upon the number of years of employment. Stated in another way we want to take steps to explore which of the four outcomes results:

- o Are exposures essentially the same for all subgroups?
- o Are exposure differences statistically significant but meaningless from a health impact viewpoint? (low exposure case)
- o Are exposure differences both statistically and medically significant?
- o Are exposure differences meaningful if postulated exposure group categories are redefined?

It is to differentiate among these possible outcomes that a sampling approach is proposed and illustrated by example. The sampling approach is summarized and the hypothesis to be tested are as follows.

- o Objective: Evaluate the exposure differences among job classifications or diesel equipment types

- Randomized (non parametric) sampling approach - i.e., no particular distribution of exposures is assumed rather a "reference distribution" based upon actual outcomes is used.

- Assume A and B the exposure groups of interest with m and n randomly selected test vehicles respectively and means X_A and X_B

- Hypothesis to be tested: Are A and B useful and valid exposure groupings?

To proceed with the test compare the difference in the means of the initially distributed groups with all alternative unique arrangements. This process is summarized as follows:

- To test hypothesis compare $\overline{X}_A - \overline{X}_B$ with distribution of all possible alternative rearrangements

- Number of unique arrangements is $(m + n)!/m!n!$

- The fraction of arrangements exceeding $\overline{X}_A - \overline{X}_B$ is a measure of the significance level of the hypothesis

- Sensitivity depends on sample size (greater number of combinations the lower the minimum level of significance inferred by the test).

For the case where we sample equally from all candidate groupings of occupations (i.e., $m = n$) the benefits of increasing the sample size are to reduce the probability that the hypothesis is unjustified. This is quantitatively shown as follows:

- For equal size groups $N = (2n)!/m!n!$

Sample Size (n)	# Possible Combinations (N)	Minimum Level of Significance (P)
2	6	0.17
3	20	0.05
4	70	0.014
5	252	0.004
6	924	0.0011
7	3432	0.00029

- Thus if goal is $P < 0.01$, a minimum of 5 samples for each group are necessary.

Obviously for uneven sample sizes across candidate groupings there is less efficiency.

TABLE 3. EXAMPLE OF NONPARAMETRIC TEST: CARBON MONOXIDE IN SCHOOL BUSES (ALL CONCENTRATIONS IN PPM)

Group A		Group B	
Vehicle No.	CO Concentration	Vehicle No.	CO Concentration
1	9.3	1	15.5
2	7.9	2	7.0
3	25.3		
4	67.9		
5	15.5		
6	13.0		
7	8.0		
8	46.1		
9	38.1		

$\overline{X}_A = 25.678$ $\overline{X}_B = 11.250$

- $\overline{X}_A - \overline{X}_B = 14.4$ and $N = 55$
- 12 combinations of $\overline{X}_A - \overline{X}_B$ groupings exceeded 14.4
- Probability that the difference of 14.4 was due to chance was 0.22. Too high to justify the hypothesis
- If 9 measurements had been made in Group B, $N = 18!/9!9! = 884 \times 55$.

To illustrate consider two populations of school buses, i.e., different manufacturers. Other factors such as route, mileage, depot, maintenance are equal. Are the populations significantly different in their interior carbon monoxide concentration - hence driver exposure. For this case only two vehicles were available of group B whereas nine were tested in group A. Results are summarized in Table 3.

In this case the probability was 22 percent that the difference in the mean values could have been the result of chance. As noted if 7 additional measurements could have been taken for group B the number of combinations used to provide comparison with the grouping mean values would have increased by a factor of 884 making the test of the hypothesis much more powerful and sensitive. We conclude the nonparametric test approach offers a useful tool in most cases.

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AN INDUSTRIAL HYGIENE CHARACTERIZATION OF EXPOSURES TO DIESEL EMISSIONS IN AN UNDERGROUND COAL MINE

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ABSTRACT

Problems exist in the United States' effort to achieve energy self sufficiency. Increasing coal production to meet President Carter's energy self sufficiency is a prime problem for the rest of the century and beyond. The use of diesels in underground coal mines has been suggested as a mining method to aid in this needed, increased production. Many questions exist about the effects on humans in such environments. NIOSH Divison of Respiratory Disease Studies has undertaken a research effort to characterize the environments of existing diesel coal mines. The results of one of the studies will be presented. Preliminary assessments of carbon monoxide, nitrogen dioxide, C_1-C_5 aldehydes and organic acids, aliphatic hydrocarbons, sulfates, total and respirable dust, and polycyclic aromatic hydrocarbons (PAH) are presented. Nitrogen dioxide and total aldehydes are suggested as possible species to quantify diesel exposure.

INTRODUCTION

Diesel powered mining equipment is extensively used today in underground metal and non-metal mines. The U.S. Bureau of Mines estimates there are more than 4,400 diesel units being used in metal and non-metal mines as of 1978 (1). The first diesel vehicle was introduced in a Western coal mine in 1946. Since that time, 33 coal mines have decided to use diesel powered equipment in their mines (2).

During 1976, NIOSH began a five-year effort to study the health implications of diesel use in coal mines. This effort includes inhalation toxicology studies, morbidity and mortality cohort studies, research industrial hygiene characterizations, and control technology assessments.

The objective of the industrial hygiene portion of this study was to characterize occupational exposures in dieselized coal mines. The benefits of the characterization are two-fold. First, meaningful toxicity testing requires knowledge of realistic exposure levels from the occupational setting. As a minimum, such levels must be known to allow valid extrapolations for dose-response relationships. Second, documenting existing levels in diesel coal mines establishes a data base for future epidemiological studies, and evaluation of control efforts.

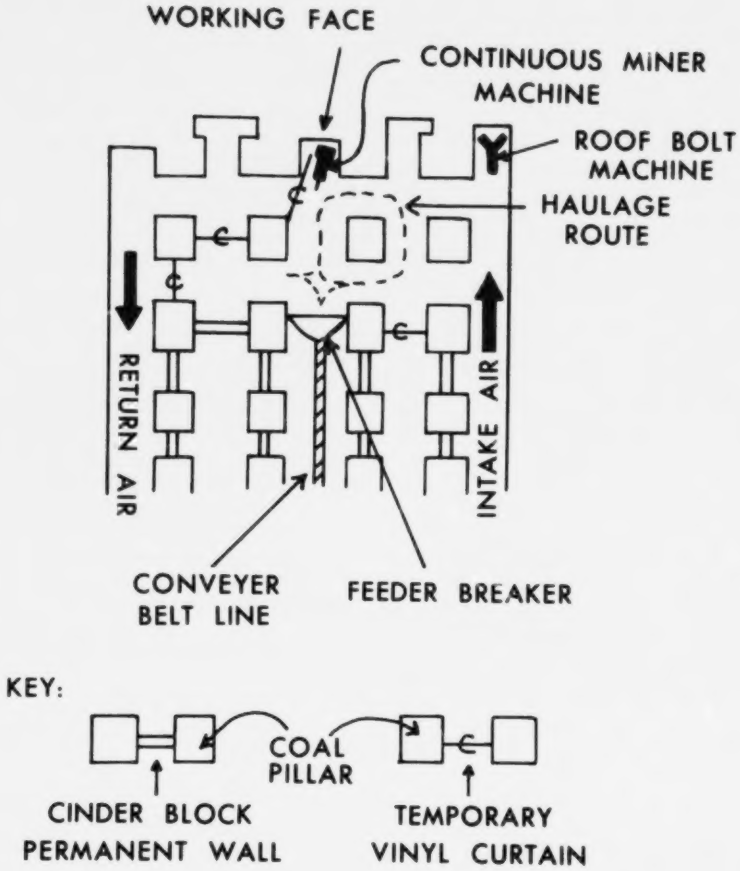
METHOD

Certain chemcial species known to be present in diesel exhaust were selected for study (3,4). These were particulates, nitrogen dioxide, carbon monoxide, carbon dioxide, cyclohexane extractable hydrocarbons, aldehydes, aliphatic hydrocarbons, organic acids, and sulfates. The collection and analytical methods are summarized in Table 1. The sampling plan was to place a sampler on each major piece of equipment within the breathing zone of the operator. Area samples were also taken in the intake air, the return air, at the haulage station, and at the feeder-breaker as located on the map in Figure 1. Samples were positioned so as not to interfere with mining operations. The samplers were put in place before mining began and collected after mining was finished to obtain full shift samples. Gas detector tubes for CO, CO_2, SO_2, and NO_2 were collected on a time-available basis.

Table 1. SAMPLING AND ANALYTICAL METHODS

Exposure	Method	Analysis
CO	Detector Tubes Ecolyzer	---
NO_2	Passive Dosimeters Ecoloyzer	Colorometric (5)
CO_2, SO_2	Detector Tubes	---
C_1-C_5 Aldehydes	Impinger	Gas Chromatograph (6)
Aliphatic Hydrocarbons	Charcoal Tubes Low Flow Pumps	Gas Chromatograph (7)
Total and Respirable Dust	DM 800 Filters WO/W Cyclone MSA Model G Pump 2.0 LPM	Gravimetric
Cyclohexane Soluble Fractions	Silver Membrane Filter B Filter WO/W Cyclone MSA Model G Pump	Sonication (8) Gravimetric
Organic Acids Combined Carboxylic Acids	15 ml Impinger NaOH 2 hrs. MSA Model G pump 1 Liter/Min.	Ion Chromatograph (9)

Figure 1. Typical mine section.



BACKGROUND

A coal mine is a dynamic system in which workers move from place to place throughout the grid of tunnels. Machinery is mounted on wheels or treads and is also mobile during a shift. Since the objective of mining is the removal of coal, the actual shape of the workplace also changes as the mine advances into uncut coal. Ventilating air is directed through the mine tunnels using cinder block walls, vinyl curtains, and auxiliary forced air systems, Figure 2. Using these techniques, the air flow pattern is changed to provide clean air to the advancing work area. The ventilation pattern is illustrated by the block diagram shown as Figure 3. Most of the mined-out tunnels in the studied mine were 20 ft. wide and 6-14 ft. high.

Coal was removed from the working face using a Jeffery electric powered continuous miner machine. Two Wagner Teletrams, model MTT-F20-18(S), were used to haul coal from the continuous miner to the feederbreaker. These teletrams were powered by Cat D330 diesel engines equipped with water exhaust gas conditioners. These vehicles are approved under Schedule 31, for use in gassy mines. At the feederbreaker, the teletrams dump the coal where it is partially crushed, loaded onto a conveyor belt, and transported to the surface. Once the continuous miner has advanced to the last roof support, it is withdrawn from that area and moved to the next working face, where the mining process continues. An electric powered roof bolter machine is then brought to the freshly mined face. Roof supports are installed to prepare for additional cuts in that area.

RESULTS

As seen in Table 2, production from the mine varied widely. Day-to-day variability in the sampler, therefore, is to be expected. Mining operations frequently encounter equipment problems requiring maintenance and causing delays. This problem is reflected in the various down times; when no coal was being mined. Ventilation rates also varied throughout the tunnel network and from shift to shift. This too is responsible for some of the sample variability. Table 2 shows the ventilation rate for the section return to illustrate this point.

Table 3 presents the results of total and respirable dust measurements. The total dust measurements are divided into area and breathing zone samples. Both kinds of samples show

Figure 2. Ventilation pattern across the working face.

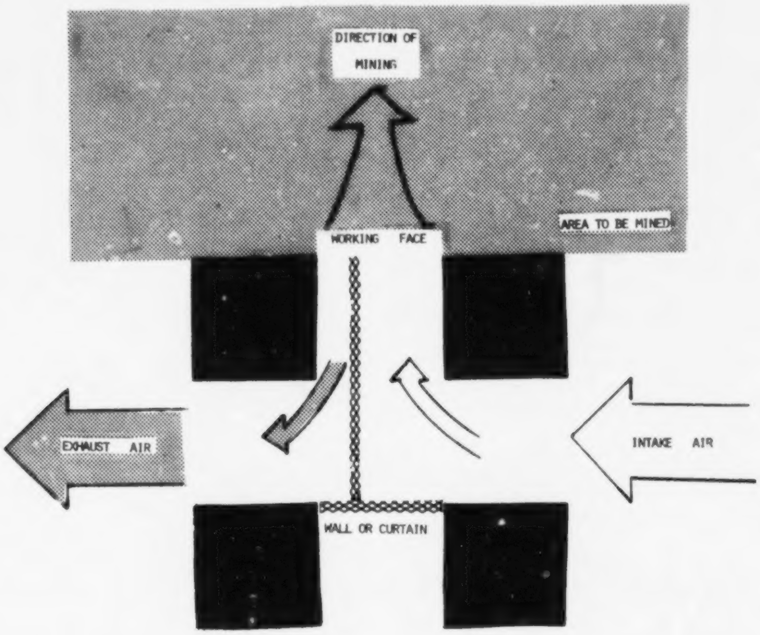


Figure 3. Ventilation network.

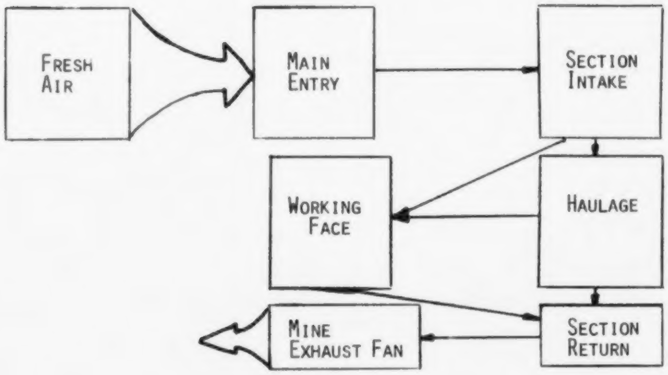


Table 2. PRODUCTION AND VENTILATION

Date	Shift	Tons Mined	Down Time Minutes	Section Return Ventilation x 1000 CFM
5/15/78	1	840	200	112.0
5/15/78	2	340	125	
5/16/78	1	600	250	81.3
5/16/78	2	840	95	
5/17/78	1	924	95	87.7
5/17/78	2	984	60	
5/18/78	1	456	335	86.7
5/18/78	2	1284	20	
5/19/78	1	864	80	74.9

Table 3. TOTAL DUST
mg/m^3

Location	No. of Samples	Geometric Mean	Geometric Standard Deviation
Intake Air	6	0.49	3.08
Haulage Area	5	1.33	2.17
Feeder Area	6	0.96	1.54
Return Air	6	1.64	4.24
Breathing Zone Samples:			
Continuous Miner Op.	7	2.15	3.63
Roof Bolt Operator	3	1.13	2.36
Teletram Operator	6	0.91	3.54

the wide variability, reflected in the geometric standard deviation, that would be predicted from the production ventilation variability. The highest area level was obtained from the return air, which is basically the exhaust ventilation for that portion of the mine. The highest breathing zone sample was obtained for the continuous miner operator, which is considered a category that normally experiences higher dust levels, whether the mine is diesel or not. Respirable dust data are shown in Table 4. The conclusions concerning total dust are the same for respirable dust, though on the average, levels are slightly lower except for return air.

Nitrogen dioxide data in Table 5 are 8-hour time-weighted averages (TWA). The NIOSH recommended standard for nitrogen dioxide exposure contains a fifteen minute ceiling value of 1 ppm. While the observed eight-hour averages were well below this value, there is no way to determine from these measurements if the limit was exceeded during any fifteen minute period. However, these results are important in evaluating the diesel contribution to mine contamination since combustion is the only nitrogen dioxide source in this mine.

Data on cyclohexane extractable material shown in Table 6 were collected on the total and respirable dust samples. Although the means are low, there are excursions above the 1979 TLV of 0.2 mg/m^3 shown in the range.

The aldehyde data in Table 7 show relatively low levels on the average; however, these may serve as an indicator of the diesel exposure.

The data in Table 8 relating other measurements show the relatively low levels of other species. The measurement of total hydrocarbons, however, was affected by the high moisture content of the mine atmosphere. Future studies should therefore avoid this methodology.

DISCUSSION

Due to the variability of mining operations, a single day's sample may not adequately reflect average concentrations for research purposes. Intercomparison of samples, however, from different work stations such as the feeder area or haulage area should be done for same day samples, to eliminate the large day-to-day variation.

Particulate levels are probably dependant on coal dust more than diesel emissions because:

Table 4. RESPIRABLE DUST
mg/m^3

Location	No. of Samples	Geometric Mean	Geometric Standard Deviation
Intake Air	5	0.42	2.85
Haulage Area	6	0.78	1.99
Feeder Area	5	0.34	2.02
Return Air	6	1.76	2.07
Breathing Zone Samples:			
Continuous Miner Op.	6	1.68	2.47
Roof Bolt Operator	3	0.91	1.97
Teletram Operator	7	0.58	1.68

Table 5. NITROGEN DIOXIDE
ppm

Location	No. of Samples	Geometric Mean	Geometric Standard Deviation	Range
Intake Air	41	0.08	3.66	.01-.30
Haulage Area	17	0.25	1.68	.09-.62
Feeder Area	14	0.34	1.52	.17-.68
Return Air	25	0.26	1.56	.14-.67
Breathing Zone Samples:				
Continuous Miner Op.	16	0.24	1.66	.11-.46
Roof Bolt Operator	10	0.03	2.80	.01-.17
Teletram Operator	15	0.21	1.52	.08-.45

Table 6. CYCLOHEXANE EXTRACTABLE FRACTION
mg/m^3

Location	No. of Samples	Geometric Mean	Geometric Standard Deviation	Coefficient of Variation
Intake Air	12	0.03	1.97	82%
Haulage Area	9	0.08	1.98	76%
Feeder Area	19	0.08	2.13	74%
Return Air	18	0.09	2.63	67%
Breathing Zone Samples:				
Continuous Miner Op.	6	0.07	2.59	70%
Roof Bolt Operator	2	0.07	1.10	96%
Teletram Operator	7	0.04	2.93	72%

Table 7. TOTAL ALDEHYDES
ppm

Location	No. of Samples	Geometric Mean	Geometric Standard Deviation	Range
Intake Air	8	2	4.45	0-68
Haulage Area	7	31	1.65	16-55
Feeder Area	15	32	3.23	0-144
Return Air	16	20	1.71	8-46
Breathing Zone Samples:				
Continuous Miner Op.	5	13	1.24	0-18
Roof Bolt Operator	2	0	1.00	0
Teletram Operator	3	11	1.56	7-17

Table 8. OTHER RESULTS AND MEASUREMENTS

Carbon Dioxide	.04-.08%
Carbon Monoxide	0-2 PPM
Carboxylic Acids	2-6 ppb
Sulfates	< 50 ug/m^3
Hydrocarbons	Charcoal tubes affected by high relative humidity
Relative Humidity	87-95%
Temperature	54° F
Pressure	28.97-29.36 in Hg.

- The cyclohexane soluble fraction is relatively constant regardless of the dust levels as seen by the coefficient of variation which does not change greatly; and
- The observed dust levels are highest near the sources of coal dust generation.

More indicative of the diesel emissions than particulate levels are NO_2 levels, which in this mine are due to combustion. However in other mines these levels may be due to blasting, thus interfering with diesel exposure monitoring. A better candidate, therefore, is total aldehydes which show the expected pattern for exposure. High levels are found around the tram and along the haulage areas and low levels are observed for the roof bolter who normally works in the fresh intake air.

SUMMARY

Using available equipment, we have begun to characterize the diesel coal mine environment. We have established ranges of contaminants that may be expected. We have identified compounds which may be used to quantify worker's diesel exposure. Finally, we intend to pursue an analysis of the correlation between production, ventilation, and observed concentrations in the dieselized coal mines.

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PANEL DISCUSSION

ON THE

HEALTH RISK ASSESSMENT OF DIESEL EMISSIONS

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Dr. Albert:

We'll move right into the panel on health risk assessment of diesel emissions. Let me introduce the panel. Next to me is Dr. William Balgord who is from Environmental Resources Technology Incorporated. Next to him is Dr. Alan Moghissi who is from the Office of Research and Development at EPA. Furthest down the line is Dr. J. Vostal from the General Motors Corporation Research Laboratory.

The ground rules for the panel are that each speaker will take five or ten minutes to make a brief statement; we will have a few minutes of discussion among the panel members themselves and then open the panel to questions and abuse from the floor. I'll start off, Dr. Balgord will be next, then Dr. Moghissi, and Dr. Vostal.

I will speak from the standpoint as Chairman of the CAG or Carcinogen Assessment Group of EPA. This is a group that was called into being by the guidelines for risk assessment that were adopted by the Environmental Protection Agency in 1976, which has provided guidance for the EPA approach to assessment of carcinogens since then. This group has been involved in doing the risk assessments for most of the agency since 1976.

I wanted to very briefly present a point of view that is embodied in the interim guidelines for risk assessment on how health risks from carcinogens are evaluated and then go on to talk about the specific issues as they are currently perceived with respect to diesel particulate emissions.

I might say that the guidelines for the assessment of cancer risks that have been adopted by the EPA are consistent with those developed by the Interagency Regulatory Liaison Group; the IRLG is made up of the principal regulatory agencies dealing with regulation of carcinogens. Within the last few months a document put out by the Federal Regulatory Council dealing with a national cancer policy also supports the EPA approach to risk assessment.

The approach to the assessment of carcinogenic risks is no different from the assessment of any other risks. It involvew two questions, How likely is the risk to occur and second, what's the impact if it does occur? Namely, How likely is the agent in question to be a carcinogen and second, If it is assumed to be a carcinogen, how much cancer is it likely to produce?

The assessment always tries to give these two answers. The first is a qualitative evaluation and the second is a quantitative evaluation. The qualitative evaluation is based on the weight of evidence, the nature of evidence, its scope and quality. A judgement is made about the weight of evidence recognizing that the best evidence consists of repeated epidemiologic studies that show the same effects under different circumstances backed up by animal studies which identify the causal agent. At the other extreme we can have a marginal response in one sex, strain, or species.

Evidence is regarded as suggestive that is based on short-term in vivo and in vitro tests, such as mutagenesis or cell transformation and initiation studies in the mouse skin. I don't believe that any regulatory action has been taken on the basis of suggestive data.

So the qualitative judgement is based on the weight of evidence approach. It clearly accepts animal tests as a surrogate for human response as well as the appropriateness of testing at high dose levels.

The second aspect of risk assessment involves the quantitative estimate of risk. It is clearly understood that at the present state of our knowledge, any quantitative assessment of risk at low levels of exposure can't be anything more than a crude ball park estimate.

Quantitative assessment requires exposure data and some means of extrapolating from high doses where observations are made, either in human epidemiologic studies or animal studies, to the low doses associated with population exposure.

In essence, the EPA, supported by the IRLG, has adopted the linear nonthreshold extrapolation model as the model of choice. This is the extrapolation model that has the most scientific support although it is generally recognized that the foundation for this or any other extrapolation model is quite thin.

The linear nonthreshold model also provides a reasonably conservative approach to estimating risks at low exposure levels. For a regulatory agency as a guardian of public health, this is an appropriate approach.

I think it is clear that the linear nonthreshold dose-response extrapolation model is the dominant concept in quantitative risk assessment; it involves the notion that there is no such thing as a safe dose and produces calculable numbers of cancer cases even at extremely low levels of exposure where large populations are involved. Indeed this concept effectively swamps out consideration of any other health risks not associated with the acceptance of a similar extrapolation model. In short, with the linear nonthreshold dose-response, if one protects against carcinogens, one can be pretty sure that one is going to be protecting against almost everything else.

It is clear that in the quantitative estimation of risks it is far better to use epidemiologic evidence than animal data because of the uncertainties in the extrapolation from animals to humans in addition to the uncertainties of extrapolating from high levels to low levels.

The assessment of diesel health risks is clearly in an evolutionary state. I think one thing that has come out of this meeting is the recentness of most of our information. At the present time we have to deal with information that has a lot of uncertainty. The impetus as you well know for concern about diesel emissions comes from positive mutagenicity tests plus the knowledge that when one combusts any organic material, carcinogens are going to be produced.

The initial risk assessment for diesel emissions had a strong element of urgency associated with it. By early 1980 very large investments were going to be committed by automobile companies, and EPA had to give some signal as to whether it regarded the health hazard in diesels as important enough to be taken into account in the planning by the automobile industry.

We were faced with the need for coming up with some sort of risk assessment strategy that could produce a defensible evaluation in a matter of months. There certainly were no animal studies available that could form the basis for an assessment.

The approach that was used was to take into account the available epidemiological evidence of lung cancer in response to exposure to combustion products since combustion products are more or less analogous to diesel exhaust particulates. This, in effect, involves lung cancer in coke oven workers, lung cancer in cigarette smokers, and lung cancer in roofers.

The point was to use these data to obtain the linear extrapolation dose-response slope for the purpose of estimating the magnitude of a cancer risk, given the exposure estimates for diesel particulates and taking into consideration the relative potencies of diesel particulates with respect to coke oven emissions, cigarette smoke tar and roofing tar.

You heard a presentation of the research results that have been prdouced in the last few months. I must say that I have to compliment the people involved. In think an extraordinarily effective and very high quality effort is being made.

I think it is fair to say that the data that has just been assembled for presentation has not really been digested. It is too early to say much about the resultss which are incomplete in any event. I can say that we are certainly looking forward to a close scrutiny of the material that is being developed as the basis for going ahead with the carcinogenic risk estimates. That is essentially what I have to say, and I pass to you, Dr. Balgord.

Dr. William Balgord:

E&RT has been involved in the diesel health issue for about the last two and a half years, dating back to our participation in the DOT fuel economy proceedings that established the 1981-85 fuel economy standards for light-duty vehicles. We have had a sustaining interest in the health implications of diesel emissions and share your impression that a great deal of information has been generated at this point. At the time we first looked into this, there wasn't very much available on the subject. But I'd like to address what I see right now as some barriers to completing the tasks necessary for arriving at a quantitative risk assessment that could be generally supported. Some of these items are rather specific - so please bear with me.

I am concerned with a general lack of representativeness regarding the way many of the (animal exposure) tests are being conducted with respect to the engine duty cycle, that is, the way the engine is operated and how that bears to the way vehicles are operated in the real world. In reading several papers, I understand that some of the engines are being operated at constant speed during the sampling periods and, correct me if I'm wrong, in no case was consideration given to collecting samples during cold-start engine operation. Duty cycles vary considerably from lab to lab as do sample collection methods. As a suggestion of this difference, I note that at one laboratory the concentration of NO_2 in the (animal) exposure chambers is on the order of 5 or 6 percent of the total NO_x as compared with concentrations that are fairly typically 15 to 20 percent in other labs. If NO_2 is a critical ingredient, along with particulate, I really feel we ought to get this together so that the results from different laboratories can be compared when we are through. I'm impressed that there have been only two engine types and vehicle types tested extensively. Admittedly when the work began, there were not that many types of diesel engines available in passenger vehicles to choose from. But this is changing. Now Volvo has one. Peugot and the Japanese are building diesels and so is a domestic manufacturer.

I think it would be important for interchange of samples to take place, particularly between General Motors (which has undertaken a very ambitious program) and EPA. In this way, we could have a feeling that the samples used during the course of the experiments had the element of comparability. It strikes me that all the particulate sampling is done with very little time delay from the moment the exhaust leaves the engine (or tailpipe in vehicle tests) to the point where the animals are exposed (or samples taken for cell studies).

I believe General Motors says this is on the order of 15 seconds. Again correct me if I'm wrong, but I don't see this as materially different from the situation in the EPA laboratory. Now this is all well and good from the standpoint of applicability to streetside exposures - a person walking near vehicular traffic along a street in downtown Los Angeles or in Manhattan - this may represent this situation. But I'm not convinced that emissions that have resided in the atmosphere for 15 minutes, a half hour, or several hours relate to the juvenile exhaust products pertaining to the diesel exposure studies described at this meeting. There is really too much opportunity for subsequent chemical transformation. This aspect needs to be considered more fully.

The engines as operated during the tests are very well attended to. We need to take account of the fact that the general population of vehicles, historically, has not had that kind of careful attention. Realistically we can't expect a car owner to bring in his diesel every month or so to have the injectors changed. The cost would be prohibitive.

A related issue is what we can expect in terms of changes in diesel fuel over the next four to six years. Currently there is quite a latitude in the ASTM specification for commercial diesel fuels. Typically, fuels do not vary much from supplier to supplier. But there is latitude within the specification for considerable deterioration in the quality of the fuel. And what with the uncertainties of the world petroleum supply, prospects are that we may have to turn to syn-fuels for at least part of the diesel's requirements. The experimental results we are now generating with very tight specification fuel - low sulfur and whatever else - may not give us an adequate picture of what these same diesel vehicles are going to emit in the near future.

I mentioned a concern with atmospheric transformations of PAH. This ties in directly to a matter of concern many of us have with the so-called artifacts of sampling. No doubt artifacts sometimes occur and have to be guarded against to avoid jumping to unwarranted conclusions. However, I would suggest that PAHs as a class undergo a series of chemical transformations in the atmosphere which eventually convert them to innocuous compounds. The concern is with how they get there. PAH, the hydrocarbon itself- as we have heard many times during these meetings - requires activation before it can express its inherent mutagenicity. On exposure to the atmosphere, the PAH may be altered in such a way that human populations will be exposed to a series of intermediates, some of which require no prior activation.

We need to progress in selecting and developing dispersion models which would apply to diesel emissions, and for that matter, to related sources. How are these to be dealt with and how are we to bring them into a suitable comparison with the contributions from diesels in cogeneration applications which are just now coming on the scene? From what I can tell, this is a subject which has not yet been addressed.

Concerning estimates of risk to human populations, we need models which adequately take into account all segments of the exposed population according to various medical histories, susceptibilities to disease, and potential susceptibility to carcinogens. Unless this is done, there is no way to justify picking out an individual as typical of the population and attempting to make a simplistic assessment of human risk applied to X million like individuals.

One particular item came up a number of times relating to the relative potency of particulate derived from gasoline or diesel engines. We need to lay out ground rules on how these apples and oranges should be compared. From what I understand of EPA's experiments with the gasoline Mustang, the carburetor was maladjusted. I don't see that as representative of a typical gasoline vehicle in today's economy. With gasoline prices having reached current levels, the average driver can ill afford a sticking choke. He'd have it fixed.

I would also encourage EPA to begin epidemiological studies addressing various populations occupationally exposed to diesel emissions.

Dr. Albert:

Thank you Dr. Balgord. Dr. Moghissi.

Dr. Moghissi:

I shall discuss the importance of fuel and fuel additives rather than the engine as sources of pollutants.

Let me define what I believe risk assessment ought to be. Risk assessment, or more precisely, environmental health risk assessment should consider the release of a toxicant, its transformation in various environmental media, the direct and indirect exposure to humans and finally, the health affects as a result of the exposure. As far as the impact of diesel is concerned, one would want to consider the components of the exhausts as well as those of the fuel -- individually analyzed and identified, their transformation in the atmosphere, and their health effects as a result of incorporation.

Obviously that is not very realistic. Recently I made a cost estimate, which showed the cost of such an investigation to be unacceptably high.

What I am going to say is the result of an effort to write regulations for fuel and fuel additives, and what I will describe are potential options or potential methods for regulating fuel and fuel additives, which have an important impact on regulating the diesel.

Starting with 1975 EPA has registered fuel and fuel additives. In 1977 the Congress -- not very pleased with the efforts of EPA -- added a section to the Clean Air Act requiring EPA to develop procedures and protocols for testing fuel and fuel additives for their health and environmental effects.

I have prepared a manuscript on this subject, which will be printed in the proceedings of this symposium. The essential features of the manuscript will be discussed here.

The fuel and fuel additives should not adversely affect the emission control system. The importance of this fact is obvious. One would also want to have the fuel and fuel additives analyzed particularly for elements other than C, H and O because presumably, unless some elements get stuck in the engine, they will appear in the exhaust. One would expect to find nitrogen in the exhaust in elemental form, as nitrogenoxides or maybe some nitrogen-containing materials such as hydrocarbons. Therefore the analysis of the original material is important.

In evaluating combustion products one has various alternatives:

Alternative number 1 is to disregard the combustion products. A number of people in this audience would appreciate this alternative. The idea being that we are already regulating the so-called regulated pollutants and if an engine does not exceed the prescribed quantity of hydrocarbons, why bother? In addition, since we know the organic content of the fuel, why bother with the analysis of combustion products? The disadvantage of this alternative is its lack of detection of a fuel that generates harmful materials.

The second alternative is the exact opposite, namely a complete chemical analysis and testing of all combustion products. This option would include transformation and health effects testing on every compound. This alternative is not very realistic because, although ideal, it is very expensive.

The alternative three would require that one could collect the exhausts and somehow try to fractionate them much like the method presented by Dr. Huisingh. For example, the exhaust mixtures are taken and fractionated into basic, neutral and acidic fractions; subsequently these fractions are evaluated for their health effects. The trouble with this alternative is that if a compound is readily decomposed in the atmosphere, it is getting the same credit as a compound that would not be readily decomposed. In fact, unless one analyzes the materials, it would be very difficult to conduct a risk analysis because one would not be able to look at the transformation and various other mechanisms that are required in the risk analysis.

The fourth alternative would set up a threshold for components of the exhaust. For example, all components falling below a certain percent (1, 0.5, 0.1, etc.) would not be considered for testing with the option that, if for whatever reason, one finds a material that is of significance, although it falls below that threshold, one would still consider it for testing.

Although there may be other alternatives, a closer look indicates that they are mostly variations to these basic four alternatives.

Once one establishes the candidates for testing, they can be subjected to the tests established by various offices of the EPA. The Office of Toxic Substances in EPA has developed certain protocols for the health effects testing. Some of these protocols are proposed, others are in various stages of preparation. After completion of these tests, one starts to make a risk analysis by establishing the quantity that is released, the air concentration, atmospheric chemical reactions, and then one selects an area where the people might be exposed to these materials such as gas stations and establishes potential acute effects. In the case of carcinogenic effects, because our present perception indicates that it has no threshold and is linear with dose, it doesn't make any difference if one exposes a smaller number of people to relatively large concentrations, or a larger population to smaller concentrations. Statistically speaking, one comes up more or less with the same number of effect.

The prerequisite is that exposure occurs at the linear and dose rate independent region. Once one does all of these, one comes up with a risk assessment for cancer.

Of course there are a number of other diseases aside from cancer. However, the mechanism for those effects would be basically the same, provided one knows at what point the threshold is and provided one can make certain assumptions relative to the shape of the dose effect curve.

As of this date the discussion centers on the triggering point of these tests. Some of these tests are rather expensive and should not be needlessly mandated. In addition, the question is, which one of these options would one want to take? Because some of the points require a resolution, I'm sure we would be very glad to hear from you. Thank you.

Dr. Albert:

Thank you Dr. Moghissi. Dr. Vostal.

Dr. Vostal:

First of all, I have to admit that all of us who have participated in the meeting during the last two days have been quite impressed by the large amount of research effort involved in the study of potential health effects of diesel emissions. Particularly, we appreciate how much work has been done on the in vitro biological activity of diesel particles by the research team of the U.S. Environmental Protection Agency. In my comments, I would like to concentrate on several items which are of importance if the objectives of all the research programs on the potential health effects of diesel emissions are to be achieved.

The U.S. EPA Mobile Sources Research Program published on January 24, 1979 formulated the governmental research objectives and indicated that

> "Recent test results and the usual carcinogenic properties of soot from incompletely burned organic material have raised the question of whether particles emitted

from diesel engines are carcinogenic. From this, two further questions arise, to wit:

1) What is the increased risk to public health if a significant fraction of new cars have diesel engines?

2) And how can the emissions from diesel cars be made less hazardous?"

Research programs aiming to answer the questions were outlined and presented to the scientific, industrial and other interested communities for their information and to receive their comments and concerns in a public Scientific Review Meeting at Arlington, Virginia, on December 12-13, 1978. We have had the possibility to comment on the appropriateness of the program during this meeting and we feel that some of our comments represent a serious criticism of the U.S. EPA research approach.

Our rationale for the risk assessment of potential health effects of diesel emissions differ from the EPA approach in at least three major research objectives:

First, we feel that mutagenicity assays or other short-term laboratory tests alone cannot serve as a sole basis for health effects assessment; second, that experimental animal inhalation models must be based on field-relevant conditions; and third, that long-term human exposure studies are the most significant factor in risk assessment.

In this meeting, we have heard the preliminary data from studies conducted by the U.S. EPA, as well as by the industry. We have learned that organic solvent extracts of diesel particulates have been extensively tested for mutagenic effects in a wide array of short-term laboratory tests with unequivocal results; we have listened to the first reports on the observed lack of significant health effects in the long-term animal inhalation experiments; and finally, we have been told in the last evening session, that the organic solvent extracts of particulates obtained from one type of diesel engine produced benign skin tumors when applied on the skin of a sensitive strain of mice in large doses, whereas extract from other engines displayed negative or only marginally positive results.

On the other hand, we have also heard presentations that bring the interpretation of the obtained laboratory data into a completely different perspective. Thus e.g., Dr. Siak presented an approach which questions if it is scientifically appropriate to use an organic solvent to extract the hydrocarbons absorbed on the core of the diesel particle. The strength of such solvent to solubilize the organic matter is many times stronger than anything found in the human body and, therefore, cannot be used to simulate mechanisms by which the biologically active compounds can be released in the organism for interaction with the sensitive cells of the respiratory system. It would seem more appropriate if - in addition to the dichloromethane extract - extracts obtained by real biological fluids were used in the skin painting testing, particularly if the results of these tests are to be used in the risk assessment process.

Similarly, I feel that the question of the maximum tolerable dose used widely in many experiments with the intention of obtaining a positive biological response in the shortest possible time needs to be reexamined. In my presentation yesterday, I showed that the calculated pulmonary dose of the diesel particulate deposits under the highly exaggerated exposure conditions exceeds the real-life situations that may be expected in the year 2000 in large urban communities, by four orders of magnitude. As a result, the exaggerated local accumulation of milligram amounts of particles may produce entirely different biological responses than those expected when only microgram amounts of diesel particulates are in prolonged contact with the sensitive respiratory cells.

Finally, data presented last night on the comparative studies of relative mutagenic and carcinogenic potencies of cigarette smoke condensate, coke oven emissions, roofing tar and organic solvent diesel particulate extract seems to indicate that the expectations that such a comparative process can be used in the risk assessment of potential health effects of diesel particulates have been premature. At least, it will not be an easy role for the governmental research teams to use the comparative data for extrapolation of well-documented epidemiology studies on carcinogenicity of cigarette smoking into a risk assessment process predicting the potential carcinogenic effects of diesel particulate exposure, if, contrary to all epidemiological experience, the relative potency of cigarette smoke condensate is completely negative in skin painting carcinogenicity tests and the benzo[a]pyrene content is lower than 1 nanogram per mg of extract. Similarly surprising is the finding that we cannot obtain a sample of the organic solvent diesel particulate extract that could be declared as a representative sample of diesel emissions. Large differences in biological activities of soot from different types of engines ranging from completely negative to clearly positive test results indicate that either our tests or our sampling procedures in the form we have in our hands today are not satisfactorily representative to provide a meaningful conclusion on the risk involved in the wider use of diesel engines on our roads. Considering the wide variations in the chemical composition of the product obtained from using identical fuel in different types of engines, as was indicated by the unbelievably large range of benzo[a] pyrene concentrations (from 2 to 1200 nanograms per mg extract in different engine samples in Dr. Huising's presentation), we have to admit that the present way of testing the diesel emissions needs to be improved before we can make any conclusions on their potential harm to human health. Similarly, the proposed comparative methods of short-term test extrapolation should not be accepted unless samples which are compared for relative potencies are compatible with the level and quality of emissions under which the epidemiological data were obtained in the exposed populations 15-30 years ago.

In this respect, I would like to return to the third point of the research objectives presented in the introduction of my comments and express my concern that the U.S. EPA approach has unjustifiably ignored a set of epidemiology data which can be important in the risk assessment process. I think that we have learned a lot about the potential health effects of diesel emissions from this afternoon's presentation by Dr. Waller from the Medical Research Council in England. Personally, I feel that in spite of the stated shortcoming, the London Transport Study remains the most meaningful and significant information for the risk assessment of potential health effects of diesel particulate that we have in our hands today, particularly since we cannot expect the results of other proposed epidemiology studies such as the investigation of the railroad workers described by Dr. Schenker today, sooner than about three years from now.

I don't want to repeat Dr. Waller's presentation, but I would like to emphasize the simple fact that despite the several thousand diesel buses and more than 10,000 fully dieselized taxicabs that have been used in the London city area during the last decades, the general incidence of lung cancer in the London population has been steadily decreasing since the 1960's, a fact which fully concurs with the conclusion of the British Medical Research Council study on garage workers exposed to high concentrations of diesel particulates. Unless we can have other significant evidence which would contradict this data, I feel we cannot abstain from its use in the risk assessment process, particularly when we realize that we have to make the important decision on the increased use of diesel engines today. The scientific community has been repeatedly assured that the automotive industry does not have any intention of increasing diesel engine use on the highways if the public health would be endangered from diesel particulate emissions, and I would like to conclude by a direct quote of Mr. E. M. Estes, President of the General Motors Corporation, published in the Environmental Health letter on November 15, 1979:

> "Despite all the research, it is unlikely that by the end of 1979 we can say flatly and without any qualification that the diesel possess absolutely no threat to human health, regardless of how remote -- if we can ever say that about anything. I do hope that we will soon be able to say that there is no unreasonable hazard -- that the benefits of using diesels in cars and light trucks outweigh any possible unsubstantiated risks."

As you see, the automotive industry depends fully on the wisdom and realistic views of the scientific community in making decisions important for the benefits of the society and all of us today. Thank you.

Dr. Albert:

Thank you Dr. Vostal. I think in view of the lateness of the hour and since the panel has already had its say, maybe we ought to open it up to the floor now. Does anybody, --

Question: Mr. Fitiansky (?): I'm Steve Fitiansky from the Environmental Science and Technology Magazine in the New York Times. First I'd like to congratulate Dr. Moghissi on his caution by quoting price figures. He's quite right. You never can tell when a member of the press is going to be located in the audience.

Dr. Vostal has presented some of his interpretations of the various results presented here and I was wondering if some of the other members of the panel could comment on whether they believe any consensus has emerged.

We've seen a lot of results which appear to be inconsistent. I wonder if you would comment on first whether there is any concensus on that mutagenicity, second carcinogenicity, and third noncarcinogenic respiratory affects.

The second question is whether you believe that mutagenicity itself is a matter of concern regardless of carcinogenicity.

Answer: Dr. Albert: Well I can start this off. I think one of the points that was raised by Dr. Vostal had to do with the importance of epidemiology. Certainly nothing can be said except to support the position. For example, if the continued evaluation of the London garage workers is negative, or even if it isn't and some good exposure estimates are generated, which don't exist at the moment, one can use the data to put an upper limit on the magnitude of the risks. Negative data on a few thousand people is not the last word when one is dealing with exposures that involve millions.

In the past, negative epidemiology data on carcinogenic responses has played a very important role in risk assessment in terms of putting a lid on any interpretations associated with animal data and essentially putting an upper limit to the response.

So far as concensus about carcinogenicity and mutagenicity is concerned, I don't think there has been an opportunity for people to consider the data. Most of it has just been presented here at the meeting and most of it I'm sure is brand new to virtually everybody in the audience. I would say there hasn't been time to consider the matter in light of whether or not there is a concensus.

Does anybody have anything to say?

Answer: Dr. Vostal: There was one important observation which came out from the discussions during this meeting. A broad spectrum of different tests was used in the U.S. Environmental Protection Agency studies to investigate the mutagenic effects of diesel particulate extracts. Surprisingly, we have seen that widely different test results were reported from testing of identical samples. Positive mutagenic effects found in one test contrasted with entirely negative results in other assays. Consequently, a tested sample could be declared mutagenic or nonmutagenic, depending on the assay used. The inconsistency of the observed mutagenic effect was not easily explained, even by the original investigators who developed the procedures and conducted the tests. I feel that it is necessary to further look into the underlying mechanisms of the postulated mutagenic effects in various biological assays before we correlate the results of short-term laboratory mutagenicity tests with the possibility of a meaningful prediction of the potential carcinogenic hazards of chemical exposures in human populations.

Answer: Dr. Albert: I think on that score it is worth pointing out that the material that was presented here really represents a pioneering effort to apply a battery of short-term tests coupled with whole animal studies to the evaluation of carcinogenic risks.

I think it really is a new effort and undoubtedly has a long way to go, but I think even on inspection of the data, it has obviously got a lot to contribute.

Answer: Dr. Moghissi: Well, I think Dr. Albert said it as well as anybody could say it. It's just a brand new field and some of the papers presented here are so new, that one has to sit down and digest them. I think the bottom line is the question if diesel engine exhaust is carcinogenic? That's really what the people are asking.

You don't have to be very smart or do many experiments to find out that there are carcinogenic materials within the exhaust of the diesel engine as they are in many other combustion products. The relevant question is not if the diesel engine causes cancer in population, rather how many cancers in addition to the naturally occurring or spontaneously occurring cases.

As I see it, the job of the science is to give at least a semi-quantitative value as to the number of those added cancers. After that the scientists are no smarter than anyone else to say if the society is willing to accept those added numbers of cancer cases. The decision of the risk at that point is no longer a scientific but a societal one that our society must decide by appropriate societal representation.

I don't think there is any question that there carcinogenic materials in diesel exhaust. You analyze it and you find that there are several papers and there are many other fully nuclear automatic materials, hydrocarbons and heterocyclic (?) materials in there, and you can imagine that it is.

Question: Speaker: IIT Research Institute. I wonder if Dr. Vostal realizes that he weakens the scientific basis of the number of his arguments by his constant insistence upon doing the requirements for doing low dose and animal testing for carcinogenics and that it has been well accepted by every organization of regulatory nature - by the National Cancer Institute's Bio (?) Program, by FTA (?) - and hopefully by EPA, that you cannot get anything but a negative answer if you do low dose testing in 50 animals. If you hope negative answers, that is the ideal way to get it, but otherwise you have to give massive doses.

Answer: Dr. Vostal: This is a very important point. However, a basic question still remains open if a short exposure to a high dose produces effects identical with the long-term breathing of extremely low concentrations. In the testing for potential carcinogenic effects, the U.S. Environmental Protection Agency proposed and used the linear, nonthreshold model as the most conservative approach to protect the innocent, and the discussion of the validity of this approach would require a longer discussion. However, the same concept should not be applied for the potential local effects of inhaled air pollutants on the respiratory system where the effects of high concentrations cannot always be found after low doses in spite of extremely prolonged exposures. This was the main reason why our long-term diesel health effects program incorporated three different levels of exposure instead of only one maximum tolerated dose used by the U.S. Environmental Protection Agency group.

Question: Dr. Kerd: I'm Dr. Kerd with the United Mine Workers. Dr. Vostal, I gathered from point three of where you said the information should come from, if that you are advocating continuing long-term human exposure studies. Would you clarify that?

Answer: Dr. Vostal: Originally, we have had plans to study the respiratory effects of the diesel particulate exposure in short-term human volunteer studies using well-controlled low-level exposure chambers, but the fact that diesel particulates were suspected as a human carcinogen prevented the realization of the project. However, other studies could be done on population groups who had been exposed to various diesel concentrations in their occupation during the past. The London Transport study, which I have mentioned briefly is only one of them. If we can identify any singular source of data which would help us to analyze potential health effects of prolonged diesel exposures, we are prepared to use the retrospective approach as effectively as possible.

Question: Dr. Kerd: I'm quite in agreement with that, however without the word retrospective in there, it becomes almost, if you read it at first glance, as though you are advocating a continued exposure in order to get the information necessary to permit the use of diesels which I think you ought to correct on your slide.

The other thing is the fact that I find that your statement by the President of this rather large corporation to which you referred, is saying that we ought not to let these things delay the use of diesels above ground. I find it very comparable to the position that co-operators are now taking and advocating the use of diesels underground in coal mines. They are not willing to see what the results are by the tests that are being made at the present time. They are so anxious to rush these instruments of evil underground, that it is almost appalling, and there is tremendous resistance building up against this use of humans as guinea pigs.

Question: Dr. Weisenberger: Dr. Weisenbeger from Cummins (?) Engine Company. I would like to ask the panel to reflect on one sentence which we see in both the IRLG documents and the CAG documents and that is the statement that the linear-threshold has the most scientific basis.

Answer: Dr. Albert: I indicated what I thought was the available evidence to support it, or maybe I didn't. One of the major lines of supporting argument is the close correlation between mutagenesis and carcinogenesis. Both appear to reflect attack by agents on DNA, the cell's genetic material, with an expression in terms of either mutation or neoplastic transformation or even cell death.

There is very strong evidence from mutagenesis, particularly in single cell organisms where one can study very large numbers of such organisms, that there is a linear nonthreshold dose response pattern. This emerged first with radiation and then with chemical carcinogens.

The second point is that there is emerging evidence from analysis of the two stage carcinogenic process in the mouse skin. Namely, that the first stage has a linear nonthreshold dose-response pattern. Those who heard Dr. Slaga's presentation must have been struck with the character of the dose response curves that he showed for skin irritation. These were very consistent with a linear nonthreshold dose response; indeed these observations have been made in other laboratories too, my own for one.

Third, there is a limited body of epidemiologic evidence which supports the possibility of the linear nonthreshold dose response in the sense that the observed evidence is consistent with it. This first emerged in the analysis of leukemia cases from atom bomb survivors; there are a few other instances where one can interpret the data as consistent with the linear nonthreshold response pattern.

Question: Mr. Fenelli: Benson Fenelli from the University of Cincinnati. From this meeting that impression has been put on carcinogenics and mutageneics of diesel exhaust. I think other after affects have been neglected. General toxicological studies have been few - effects status have not been satisfactorily done or they have not even been attempted. Could you comment, other than the carcinogenic and mutagenic study, why don't we center our attention to more health effects in general?.

Answer: Dr. Vostal: We have seen during the discussions in this meeting that attention has been also paid to other potential health effects of diesel emissions, rather than concentrating only on mutagenesis and carcinogenesis. Thus, our laboratory presented several papers yesterday that discussed possible changes in lung function, morphology and biochemistry. We have studied the effects on enzymatic composition of tissue cells and blood plasma and we have other ongoing studies centered on potential effects in immunological response. As you may remember, so far we have not found any effects after nearly twelve months of exposure that would be of serious concern or specific for diesel particulates.

Question: Speaker: I have two comments. The first relates to the comments you offered Dr. Albert on the linear nonthreshold model which certainly would agree with most of what you said but I think one comment was perhaps subject to misinterpretation. You said that the use of the model swamped out any other risk projected using alternative models and thus it would protect against anything else. In essence, to paraphrase what you said, I think that you offered that as related to that specific agent. Clearly we have I think in this specific situation an option in which we are not looking just at control of the specific agent, but we are forced to make comparisons to other risks, risks that may not be adequately described with the linear nonthreshold model, and so I think the situation becomes very much more complex. It falls really on the heels of the last gentleman's comment as we talk about effects that may be produced by other agents.

I think that is necessary to keep this in mind as we look at the use of the linear nonthreshold model, especially in comparative assessments.

The second is that it seems to me that one of the lessons that comes through the last three days is something that is perhaps related to the study of other materials. We've heard data indicating very clearly the presence of potent mutagens within diesel exhaust extracts; however, the majority of the data at hand today, if not negatives, are at least indicating the extent to which diesel exhaust particles are relatively weak carcinogens.

I think this is important as we look to the issue of other materials that are introduced into our environment and that are in our environment now, and may be introduced in the future. As most of us I think are aware, we have to exercise a great deal of caution in the interpretation of short-term screening tests in terms of mutagenicity. They in themselves cannot be the basis for taking final action on a product whether it should or should not be present. I think this certainly concurs with your comments and several of the comments of other panel members.

Answer: Dr. Albert: I would agree. This relates to the question whether mutagenicity is enough to raise a concern about regulatory action. I think the essential ingredient that is missing from mutagenicity data derived from short-term tests is the issue of whether or not there is penetration of the agent in question to the germ cells.

There really hasn't yet been a coherent approach to the regulation of mutagens as with carcinogens. Guidelines for assessment of mutagens in the Environmental Protection Agency are being attempted at the present time.

Question: Mr. Culver: Allen Culver from Research Triangle Institute. I have been amazed in the past six or seven years by the amount of cooperation that has occurred between analytical chemists and biologists in investigating problems such as the diesel particulate problem, and I also respect very much Dr. Vostal's comments; and in order to help resolve some difficulties in that area, the ball can be thrown a little now to the chemists.

We know what compounds are heavy-duty carcinogens and we know what they are; we have a little bit of an idea as to how much is required to produce a tumor. What we don't have a good idea of at the moment is a good quantification of the presence of these compounds in extracts from diesel particulates; however, the extracts may be made.

I think that the chemist serves a very important function while the biological development lags and is being developed; the chemist can serve a role in trying to quantify the important carcinogens present in the diesel emissions under various conditions. I think that may now be a mandate in this field of research.

Answer: Dr. Moghissi: We discussed that subject among some of our colleagues, and you will find differences of views in this area. I happen to agree that the chemistry has to go hand in hand with the biology; you had better know what you are feeding to your animals or letting your animals inhale.

There are, however, some people who believe that you can simply take a mixture and do biological experiments. There is some justification to this approach, and in certain well specified cases one comes up with satisfactory answers.

I happen to believe that in the long range there is nothing better than systematic separation and biological evaluation of each compound.

Question: Dr. Albert: Are there any other comments?

Question: Mr. Friedman: Bob Friedman, Bureau of Mines. Underground we are trying to ease the problems of chemist, biologist, and so forth doing analysis, by going to control measures; there is some hope of controls underground. We are beginning to see the light at the end of the tunnel.

Now I know that GM is, for example, and probably other companies as well, experimented with control devices such as particulate removal on exhaust. How well they are going with that right now? Are you willing to say?

Answer: Dr. Vostal: The automotive industry studies intensively all possible devices for particulate removal or exhaust modification. However, the question is entirely technical, and I do not feel that I am the proper person to answer it.

Question: Dr. Albert: Anybody know how well...

Answer: Dr. Klimisch: Dick Klimisch, GM Research Laboratories. I am not a spokesman for GM on this subject, but I will answer the question since no one else is around now who can do it better. There are certain problems in applying the underground control systems to the aboveground situation. Obviously we are working on traps and things like that, but we can't use these gigantic water scrubbers underground or we will lose the fuel economy advantages that moved us toward diesels in the first place. The biggest problem currently is the interaction between nitrogen oxides and particulates, that is, trying to get both of those emissions down at the same time. We are making progress but we are not yet there.

Question: Dr. Albert: We'll take one more.

Question: Mr. Peters: Bill Peters, MIT. I'd just like to thank Dr. Balgard for drawing our attention to the need to consider the operating conditions of the engines and also the fuel type; the latter could be very important in defining the optimum utilization for various synthetic fuels, fuels based not only on coal, but also on shale and perhaps even biomass, and so I just appreciate your mentioning that.

Question: Dr. Albert: This will be the last.

Question: Mr. Louflin: Dennis Louflin. There is one thing that I am concerned about. What is the possibility of inaccurate interpretations of risk? I think there are some dangers that people don't really discuss. For example, in the regulatory council statement and the IRLG statement, there is some indication there that the screening is set up to overestimate risks. I think one has to be mindful (?) that one ought to protect the work force but only to the degree that one does not do that to an extreme, in which case you tend to overstate risks. I think this could be very significant.

For example, if control technology is adapted for diesels in the coming years because of the risks compared to automotive, and if a hasty decision is made, then it seems as though the public at large tends to suffer.

I think in the IRLG screen they don't really address what the consequences are of overstating the risks, and I think it might be significant because I noticed in the IRLG screen that there is no mechanism for correcting a false identification of harmful agents such as carcinogens. I think that in this carcinogens issue that it is actually in comparing things to other indexes like coke-oven (?), it's not going to happen by helping the diesel issue that it isn't taken lightly because perhaps substitute agents can be more harmful. Dr. Albert you might comment on the IRLG screen. I think to some degree it does overestimate. Have they looked at the risks of overestimating?

Answer: Dr. Albert: Well, I think that there hasn't been any major regulatory action taken on the basis of short-term tests. Conformatory studies in animals for carcinogenicity are required. But it seems to me that you have a point. I think there is a tendency in the risk assessment business to err on the conservative side, and there obviously has to be a limit to that. This is a matter of balance and judgment given the present state of our knowledge about these matters.

APPENDIX

REGULATORY OPTIONS FOR THE DEVELOPMENT OF HEALTH AND ENVIRONMENTAL TESTING OF DIESEL FUELS AND FUEL ADDITIVIES

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It is well known that mobile sources have a significant impact on air quality. Traditionally, the U.S. legislation has been directed at the automobile engine to reduce the quantity of air pollutants. However, in recent years, the significance of fuel and fuel additives in the reduction of air pollution has been recognized. The Clean Air Act and its recent amendments have made provisions for registration and testing of fuel and fuel additives. The U.S. Environmental Protection Agency (EPA) has an ongoing registration program since 1975 and has registered several thousand materials. The 1977 amendment to the Clean Air Act required the development of regulations for testing of fuel and fuel additives including deadlines to develop protocols for environmental and health tests and criteria for testing. The discussion of the legislative mandate of the EPA is beyond the scope of this paper. This paper limits its scope to options for these tests and the framework for the development of appropriate regulations.

Health and environmental tests are advantageously divided into two groups. One group would identify compounds or mixtures of compounds to be tested, and the other group would specify which tests are to be conducted on those compounds or mixtures of compounds identified in the first group of tests. As a matter of convenience, the compounds identified in the first group of tests, henceforth, will be referred to as candidate test materials (CTM).

The first group of tests, as mentioned previously, may lead to the identification of CTMs through various mechanisms; however, these tests also may be conducted to assure compliance with present regulations such as emission of the so-called regulated pollutants.

CHEMICAL ANALYSIS AND FUEL PROPERTIES

It is readily understood that the knowledge of elemental composition of fuel and fuel additives is useful in evaluating their environmental impact. In this case, one is not concerned with C, H, or O; rather the heteroatoms such as N, S, and P and trace metals are of concern. Because most elements are converted to their highest oxidation stage during the combustion process, exhaust will consist of CO_2, H_2O, N_2, NO_x, SO_2/SO_3, P_2O_5 and oxides of various metals. The availability of the elemental composition of the fuel would therefore permit an estimate of environmental release of various pollutants. Additionally, various analytical techniques such as atomic absorption spectroscopy permit rapid and comparatively inexpensive analysis of any trace elements down to 0.01 percent.

In addition to elemental composition, the availability of chemical composition of fuel and fuel additives is important. For example, the evaporative emissions are significantly impacted by the composition of the fuel mixture. Also, the presence of polynuclear aromatic compounds in significant quantities would be undesirable; therefore, a minimum knowledge of chemical composition is both desirable and necessary.

Among fuel properties of concern are distillation curve and cetane number (octane number for gasoline) because they impact the proper operation of the engine and thus impact the environmental releases of pollutants.

EMISSION TESTING

Emission testing, as proposed or promulgated in EPA regulations, refers to testing automobiles for compliance with regulated pollutants, namely CO, NO_x, and total hydrocarbons for gasoline engines and particulate emissions for diesel engines. Emission testing does not lead to identification of a specific pollutant; rather, it assures that the total emissions of an automobile is not adversely affected by the introduction of new fuel, a new additive, or a new combination of known fuel and fuel additives.

Because the evaporative emissions can be greatly influenced by the fuel and fuel additives, evidence should be presented that a new fuel or a new additive will not cause excessive evaporative emission from an automobile which has met the standards. Evaporative emissions can be sometimes predicted on the basis of certain properties of the fuel. Several oil companies have developed theories to predict evaporative emissions and have experimentally tested these theories. Because of the progress in this field, it is sometimes possible to consider predictive models as substitutes for testing for evaporative emissions provided they have been confirmed by at least a limited degree of experimental data.

COMBUSTION PRODUCT TESTING

As mentioned previously, the EPA has proposed or promulgated limits of emissions of carbon monoxide, nitrogen oxides, hydrocarbons (HC), and particulates from diesel engines; however, health and environmental impact of automobile emissions depend upon the composition of HC (and particles from diesel). For example, it is readily conceivable that the HC fractions resulting from two different fuels may have very different health effects, although both emit the same quantity of HC per vehicle mile. The purpose of combustion product testing should be to identify those materials which will be considered for testing for their environmental and health effects. To facilitate the discussion of these alternatives, a new term in the combustion products is defined. This fraction, emission products other than common oxides (EPOTCO), consists of the entire exhaust with the exception of oxides of carbon, nitrogen and sulfur; water; nitrogen; oxygen and noble gases.

Several alternatives may be considered in the identification of CTM.

Alternative 1 - Disregard Combustion Products

It is well-known that the collection and identification of combustion products are difficult and not standardized.

Combustion products in EPOTCO are numerous and their production depends upon so many engine parameters, such as engine temperature and air-to-fuel ratio, that great difficulty would be experienced in developing a standard procedure for the identification of EPOTCO. Because the total quantity of pollutants of concern is already limited, EPOTCO would not be analyzed or identified and would not be considered in the health and environmental testing program.

The advantage of this alternative is its simplicity and ease of regulatory process. Its major disadvantage is a disregard for potentially harmful materials in the combustion products.

Alternative 2 - Complete Analysis

All compounds in EPOTCO are analyzed to the lowest level of presently available analytical techniques. In so doing, one would choose specific engine cycles which are representative of the current year models of the automobiles. Each one of the identified compounds would be subjected to environmental and health effects testing.

The advantage of this alternative is the comprehensive analysis of the exhaust and the possibility of considering every single identified compound for health and environmental testing; however, the required extreme care in controlling engine parameters, sample collection, and the unusually high sophistication in the analysis make such a system unrealistic and expensive.

Alternative 3 - Collection of the Exhaust, But No Analysis

Toxicity tests will be conducted with the entire exhaust or fractions of the exhaust such as gaseous and other fractions. For example, particulates from a diesel engine would be collected and used for toxicity testing, either directly or after fractional separation.

Also, this alternative suffers from major disadvantages. The presence of one compound with sufficient acute toxic properties would make it difficult and occasionally impossible to observe potential chronic effects. In addition, materials with widely different environmental persistence would be given equal ranking in their toxic effects. For example, if a compound is readily decomposed in an aqueous medium in the environment within a short period of time, it would be given equal consideration with a compound which is persistent for a long time. Other disadvantages of this alternative include the extreme difficulty in collecting a "realistic" sample. Compounds which, because of dilution in the atmosphere, would not react with each other may react under conditions of toxicity testing and generate new materials having significantly different toxicity properties than those originally present.

Alternative 4 - Partial Analysis

Only those compounds which are above a certain percentage of the total EPOTCO fraction will be identified. This alternative is a more simple and less expensive version of Alternative 1. Although changes in engine parameters may cause differences in the composition of the EPOTCO fraction, it is anticipated that many compounds will appear above the threshold level regardless of the engine parameters, provided these parameters are kept within realistic range of conditions. For example, all compounds appearing in concentrations of more than 0.1 percent in the EPOTCO fraction would be tested.

The advantage of this alternative is that it covers a major part of EPOTCO fraction without the requirement of a comprehensive analysis. The disadvantage of this alternative is the possibility that highly toxic materials appearing in concentrations below the threshold level will not be identified and thus will not be considered for further testing.

The choice of the threshold level is significant in that it establishes the number of materials for further testing. For the gasoline engine, a level of 1 percent by weight in the EPOTCO fraction would cover about 20 compounds, a 0.5% by weight, about 35 and a level of 0.1 percent by weight, about 70 compounds (1-12). The number of compounds for diesel are unknown at this time although their number should not be significantly different from that of the gasoline engine. Regardless of threshold chosen, there may be compounds below the threshold which could be of potential hazard and should be identified and tested.

CANDIDATE TEST MATERIALS (CTM)

Once various components of the fuel, fuel additive, and combustion products are identified, they become potential candidates for further testing. These CTMs could be a simple compound or, depending upon the selection process, mixtures of various compounds. Obviously, one has to establish, at least on a semiquantitative basis, potential exposure of the public before a CTM goes through a series of tests usually associated with the evaluation of a compound.

BIOLOGICAL TESTS

The Office of Pesticides and Toxic Substances (OPTS) of EPA has proposed various rules for biological testing of pesticides and toxic chemicals (13). Many of these tests are generally applicable tests for toxicity evaluation of a chemical. For example, acute inhalation toxicity tests and chronic carcinogenicity tests can be conducted with pesticides as well as with components of fuels. However, as a general rule, the route of exposure to fuel and fuel additives and their combustion products is usually dominated by inhalation and thus this route should predominate. Also, because of potential skin exposure as a result of spillage, this route should be considered. A convenient way to categorize biological and environmental tests is as follows:

Acute Toxicity Testing

These tests are designed to evaluate potential hazard of fuels and fuel additives on a localized and concentrated basis. For example, potential toxic effects of fuels and fuel additives to the customers of self-service gas stations will be evaluated using the results of these tests. Acute toxicity tests are well standardized and have been used for registration of pesticides and are being proposed for premanufacturers testing of toxic substances. On the basis of potential exposure routes, the following tests could be considered:

- Acute Inhalation Toxicity
- Subchronic Inhalation Toxicity
- Acute Dermal Toxicity
- Subchronic Dermal Toxicity
- Eye Irritation

Chronic Toxicity Testing

These tests are designed to evaluate the long-term potential health impact of fuel and fuel additives and their combustion products. Protocols for these tests have been described in proposed guidelines for registration of pesticides and are being developed for toxic substances. Examples of these tests are:

- Mutagenicity
- Teratogenicity
- Oncogenicity
- Other Chronic Effects

Environmental Testing

The purpose of environmental testing is to establish the indirect exposure of population to fuels and fuel additives and the resultant products of their combustion. Examples of these tests are:

- Soil Degradation
- Atmospheric Stability
- Plant Uptake Studies

Because the exposure to these matrials predominates in the urban areas, plants grown in the urban environment should be considered for testing.

RISK ANALYSIS

The biological and environmental tests are conducted to avoid unnecessary and unwarranted human health risks. Therefore, at least a semiquantitative analysis should be conducted to evaluate potential effects of a fuel or fuel additive. Methods for risk analysis are being developed by various offices of EPA and by several other U.S. agencies and other organizations. The EPA, along with several other agencies, has published its cancer policy (14) and quantitative techniques for radioactivity have been in operation for many years (15). It is fully recognized that a quantitative risk analysis for many toxicants may be possible only after extensive testing, or may be unfeasible because of economic reasons. This problem is, however, common to all toxic chemicals and is being addressed by EPA and a number of other agencies. Once a policy decision is made as to the approach, it is applicable to all toxicants including fuel and fuel additives.

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CONCLUSIONS

There are a variety of options for regulating fuels and fuel additives. The selection of an option should be based upon sound economic considerations and on potential risk to the population. In many cases, one can readily make appropriate decisions solely on the basis of a chemical analysis. In other cases, toxicity data may be available and the cost of regulating a fuel or an additive may consists of a literature search and application of sound scientific models to predict potential hazards of fuels, fuel additives, and their combustion products. In other cases, it may be necessary to conduct a series of biological tests. Fortunately, protocols for biological tests are either developed or are being developed by various offices of EPA. The regulations should spell out methods for exempting a fuel or an additive for certain tests after exposure is negligible.

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